THE REGULATORY NETWORK CONTROLLING NATURAL COMPETENCE FOR DNA UPTAKE IN *VIBRIO CHOLERAE*

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THE REGULATORY NETWORK CONTROLLING NATURAL COMPETENCE

FOR DNA UPTAKE IN *VIBRIO CHOLERAE*

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This is for you, Ivan, Mom and Dad.
Thanks for always being there for me,
and for believing that I can accomplish great things.

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TABLE OF CONTENTS

Page

LIST OF TABLES

LIST OF FIGURES

LIST OF SYMBOLS AND ABBREVIATIONS

AI	autoinducer
AI-2	autoinducer-2
cAMP	cyclic adenosine monophosphate
Amp	ampicillin
ASW	artificial sea water
BLAST	Basic Local Alignment Search Tool
bp	base pair
CAI-1	cholera autoinducer-1
CBP	chitin binding protein
CCR	carbon catabolite repression
CDC	Centers for Disease Control and Prevention
CFU	colony-forming unit
ChiPR	chitin regulated pilus
Cm	chloramphenicol
CRE	competence regulatory element
CRP	catabolic repressor protein
CTX	cholera toxin
CytR	cytidine repressor protein
DNA	deoxyribonucleic acid
dsDNA	double stranded deoxyribonucleic acid
ssDNA	single stranded deoxyribonucleic acid
DUS	DNA uptake sequence
GpbA	GlcNAc binding protein
GlcN	glucosamine
GlcNAc	*N*-acetylglucosamine
c-di-GMP	Bis-(3'-5')-cyclic dimeric guanosine monophosphate
$Gram^-$	gram negative bacteria
$Gram^+$	gram positive bacteria
HCD	high cell density
HGT	horizontal gene transfer
HK	histidine kinase
IM	inner membrane
IPTG	isopropyl-β-D-thiogalactopyranoside
Kan	kanamycin
LCD	low cell density
LB	Luria-Bertani broth
LM	Luria-Marine broth
MMR	mismatch repair system
µg	micrograms
mM	millimolar
µM	micromolar
mL	milliliter
MSHA	mannose-sensitive haemagglutinin pilus
OD	optical density
OM	outer membrane
PCR	polymerase chain reaction
Qrr	quorum regulatory RNA
QS	Quorum sensing

RBS	ribosome binding site
RLU	relative light units
RNA	ribonucleic acid
mRNA	messenger ribonucleic acid
sRNA	small ribonucleic acid
RNAP	RNA polymerase
Str	streptomycin
TCP	toxin-coregulated pilus
UTR	untranslated region
WT	wild type

SUMMARY

The bacterial pathogen *Vibrio cholerae* is responsible for ongoing cholera outbreaks in Haiti and elsewhere. Association of *V. cholerae* with the human host is responsible for fatal disease, but the bacteria also reside as natural inhabitants of aquatic environments, commonly attaching as biofilms to chitinous surfaces of copepods and crabs. Prior studies in *V. cholerae* demonstrated that competence for genetic transformation, a mechanism of horizontal gene transfer (HGT), requires the TfoX regulator protein that is triggered by chitin, and the HapR transcription factor that is made in response to quorum sensing (QS) signals produced by *V. cholerae* and *Vibrios*. To define regulatory components connecting extracellular signals to natural competence, I first demonstrated that QS molecules produced by *Vibrios* within multi-species chitinous biofilms are required for DNA uptake by *V. cholerae,* confirming the critical role of QS signals in HGT. Second, I identified by transposon-mutagenesis a new positive regulator of competence, CytR (cytidine repressor), only studied prior in *E. coli* as a regulator of nucleoside scavenging. Specific mutations in *V. cholerae* CytR impaired expression of competence genes and halted DNA uptake; and the addition of exogenous cytidine had similar affects as predicted in *E. coli*. *V. cholerae* and other competent *Vibrios* encode TfoX, HapR, and CytR, although none of these regulators directly controls genes coding for the DNA uptake apparatus. Thus, these results have uncovered a regulatory network, likely used by many *Vibrios*, that contains additional factors linking several extracellular chemical molecules (cytidine, chitin, and QS signals) to DNA uptake. My study has begun to define a molecular mechanism by which both environment and genetics contribute to genome evolution for this important marine pathogen.

CHAPTER 1

LITERATURE REVIEW

1.1. Introduction

Vibrio cholerae may be most notable as the causative agent of major outbreaks of diarrheal disease cholera in locations such as Bangladesh, Kolkata, and more recently Haiti (42), but this bacterium is also a natural inhabitant of aquatic environments. Within marine and brackish water systems *V. cholerae* commonly forms biofilms on abiotic and biotic surfaces, such as planktonic organisms, chitinous chironomids (non-biting flies), and exoskeletons of copepods and crabs (72, 93, 169). Association with particulate matter likely aids in transmission of this and other waterborne pathogens (49), however, attachment to chitinous surfaces also provides physiological benefits to *V. cholerae*. In particular, chitin initiates a developmental program of gene expression allowing *V. cholerae* to be naturally competent to take up naked DNA from the environment (118). New genetic material may also be obtained by conjugation (via plasmids) and transduction (via bacteriophages), but when naked DNA is successfully incorporated into the chromosome and provides novel behavior to the recipient, this process of horizontal gene transfer (HGT) results in “transformation” of the recipient. Natural transformation was first described in 1928 by Griffith in *Streptococcus pneumoniae* (67), and since then it has been well-studied in several other bacteria (57, 154).

Recent studies in *V. cholerae* reveal that chitin is one of several signals to activate regulatory systems that induce natural competence. In particular, genetic evidence

supports a role for a chitin utilization system, a quorum sensing system (QS), and a nucleoside scavenging system in the control of genes that encode components of the "competence apparatus" (4, 22, 118). Carbon catabolite repression (CCR), a global response to glucose starvation, impinges on all three systems (4, 22, 109). It is appreciated by studies of other naturally competent microbes that acquisition of extracellular DNA may be used for genome repair, nutrition, as well as for HGT (for reviews see (57, 146, 163)); and recent studies described here suggest this may be the case in *V. cholerae* as well. Since Meibom *et al* first documented natural competence in *V. cholerae* in 2005 (118), several other *Vibrio* species have been shown to be capable of taking up extracellular DNA (40, 70, 139). It is likely that conditions under which other *Vibrio* species are also transformable remain to be identified. Finally, although many regulatory systems promoting this process have been identified, much work remains in defining the genes and regulatory connections linking extracellular signal recognition to production of and uptake by the transformation apparatus, as well as the fate(s) of the DNA acquired by this process.

1.2. The competence apparatus

The movement of extracellular naked DNA from outside the cell to the cytoplasm requires a complex competence apparatus. Pioneering studies have elucidated many of the structural components of the apparatus in gram positive ($Gram^+$) bacteria, such as *Bacillus subtilis* and *Streptococcus pyogenes*, and also ($Gram^-$) negative bacteria, like *Neisseria meningitis* and *Haemophilus influenzae,* which are more closely related to *V. cholerae* (reviewed in (57)). The structure and organization of the $Gram^-$ competence apparatus will be used as a schematic model to describe the putative analogous apparatus likely used by *Vibrios*, with an emphasis here on *V. cholerae* (Fig. 1.1). Future

studies will undoubtedly determine the role of each of these components in *V. cholerae* and unique characteristics that distinguish the *Vibrio* apparatus from that of other bacteria.

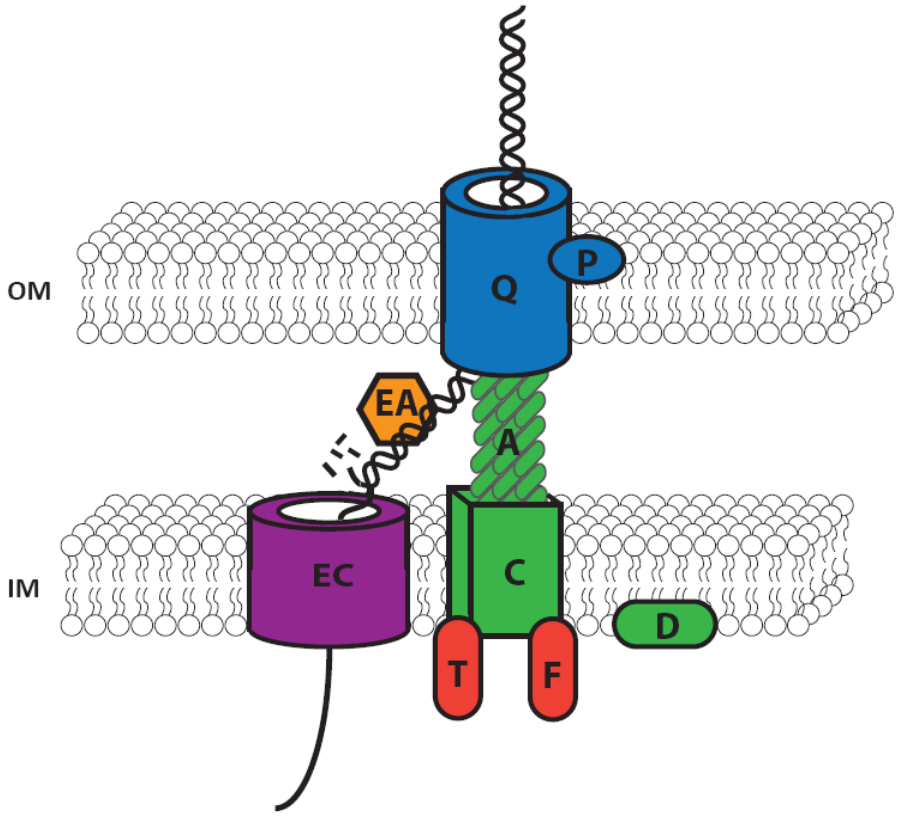

Figure 1.1. Schematic model of *Vibrio cholerae* competence apparatus. Incoming dsDNA is transported across the outer membrane (OM) through the PilQ secretin aided by PilP (blue). The pseudopilin is composed of PilA subunits processed by the prepilin peptidase (PilD) and anchored to PilC (green), with assistance from the PilT and PilF NTPases (red). Periplasmic dsDNA associates with the ComEA DNA-binding protein (orange) and then one strand is degraded by extracellular nucleases. The ssDNA accesses the cytoplasm through the inner membrane (IM) ComEC channel (purple).

The competence machinery (Fig. 1.1) delivers extracellular DNA through both the outer membrane (OM) and inner membrane (IM) of Gram$^-$ bacteria. *H. influenzae* and *Neisseria gonorrhoeae* both restrict uptake to species-specific DNA, by binding to DNA uptake sequences (DUS), of 9-10 nucleotides, respectively, which are enriched in the

respective genome of each bacterium. Recently, the ComP protein in *N. meningitidis* has been identified as a likely DUS receptor that displays higher binding affinity for specific-specific sequence than for non-specific sequence (32). *V. cholerae* does not appear to restrict uptake to species-specific DNA, as Suckow *et al* have demonstrated the capacity of *V. cholerae* to take up DNA from other bacteria, such as *Escherichia coli*, into the periplasmic space between the IM and OM (165). Consistent with these observations and the lack of specificity for DNA by *V. cholerae*, *Vibrios* do not appear to encode an analog of ComP.

1.2.1. The competence secretin and channel

Extracellular double stranded DNA (dsDNA) is delivered across the outer membrane (OM) by the secretin channel (Fig. 1.1). In *N. meningitidis* (48), PilQ, which also serves as the type IV pilus secretin, forms a multimeric structure in the OM channel, with assistance from the PilP "pilot" protein required for secretin assembly. The secretin allows passage of extracellular dsDNA into the periplasm where it can then access an inner membrane (IM) channel. In *V. cholerae*, the ComEC protein encodes the IM channel, as a Δ*comEC* mutant takes up DNA into the periplasm via PilQ, but does not deliver it into the cytoplasm (165) (Fig. 1.1). *V. cholerae* and the other naturally competent *Vibrio* species encode a *comEC* and also *pilQ-M* operon orthologous to that in other Gram$^-$ bacteria (Table 1.1). *V. cholerae* Δ*pilQ* mutants are not naturally competent, which is consistent with their predicted role in DNA transport (118). The roles of PilM, PilN and PilO in bacteria remain poorly understood.

Table 1.1. Predicted and known *Vibrio cholerae* competence machinery and regulatory genes, and their orthologs in other *Vibrio* species

Annotation	*V. cholerae* N16961	*V. parahaemolyticus* RIMD	*V. vulnificus* YJ016/CMCP6	*V. fischeri* ES114
OM transport				
pilQ	*vc2630*	*vp2746*	*vv2990/vv_1381*	*vf2293*
pilP	*vc2631*	*vp2747*	*vv2991/vv_1380*	*vf2294*
pilO	*vc2632*	*vp2748*	*vv2992/vv_1379*	*vf2295*
pilN	*vc2633*	*vp2749*	*vv2993/vv_1378*	*vf2296*
pilM	*vc2634*	*vp2750*	*vv2994/vv_1377*	*vf2297*
pseudopilin				
pilA	*vc2423*	*vp2523*	*vv2778/vv_1626*	*vf2185*
pilB	*vc2424*	*vp2524*	*vv2779/vv_1625*	*vf2186*
pilC	*vc2425*	*vp2525*	*vv2780/vv_1624*	*vf2187*
pilD	*vc2426*	*vp2526*	*vv2781/vv_1623*	*vf2188*
DNA binding				
comEA	*vc1917*	*vp0922*	*vv1109/vv_0017*	*vf0801*
IM transport				
comEC	*vc1879*	*vp0981*	*vv2359/vv1_2084*	*vfa0423*
NTPases				
pilU	*vc0463*	*vp2614*	*vv2872/vv_1527*	*vf0432*
pilT1	*vc0462*	*vp2615*	*vv2874/vv_1526*	*vf0431*
pilF	*vc1612*	*vp1752*	*vv1491/vv_2773*	*vf0627*
regulators				
crp	*vc2614*	*vp2793*	*vv3050_vv1_1318*	*vf2280*
tfoX	*vc1153*	*vp1241*	*vv1446/vv1_2820*	*vf0896*
cytR	*vc2677*	*vp0252*	*vv3013/vv1_1358*	*vf2275*
hapR	*vc0583*	*vp2515*	*vv2770/vv1_1634*	*vf2177*
qstR	*vc0396*	*Vp2710*	*Vv2954/vv1_1429*	*?*

1.2.2. Periplasmic DNA binding and degradation – ComEA and DNase

Both Gram$^+$ and Gram$^-$ bacteria utilize a dsDNA binding protein, ComEA, that serves as a non-specific DNA receptor assisting in delivery of DNA to the IM channel (Fig. 1.1). In *N. gonorrhoeae*, ComEA (ComE) is periplasmic, while ComEA of naturally competent *Thermus thermophilus* contains a predicted transmembrane domain supporting a model that it is an IM protein (37, 63, 142). Each *Vibrio* is predicted to encode a ComEA ortholog (Table 1.1), and a *V. cholerae* Δ*comEA* mutant is not transformable (118). Because a DUS receptor remains to be determined and is perhaps not utilized for uptake in *Vibrios*, any interplay between ComEA and a putative DUS remains

speculative in *V. cholerae*. Based on other Gram$^-$ naturally competent bacteria, dsDNA that is delivered through the OM and then recognized by ComEA, is processed by periplasmic DNases that degrade one strand of the DNA. In other bacterial systems, only single stranded DNA (ssDNA) enters the cytoplasm through the IM channel while the other strand is degraded. *V. cholerae* encodes two periplasmic DNases studied for their role in competence, Dns and Xds. Genetic evidence indicates that Dns is the major enzyme required for degradation; as Δ*dns* mutants are hypercompetent for transformation while Δ*xds* mutants behave similarly to an *xds*$^+$ strain (23). Interestingly, both nucleases also alter biofilm formation consistent with a role for extracellular DNA in biofilm structure (155). In both Gram$^-$ and Gram$^+$ bacteria, ssDNA is ultimately the form that enters the cytoplasm via the ComEC channel although this has not been experimentally validated for *Vibrios*.

1.2.3. The competence pseudopilin and accessory proteins

A pseudopilin structure is thought to assist in delivery of exogenous DNA to the IM channel by dynamic assembly and then disassembly (Fig. 1.1). By analogy to the related Type IV pilus, the pseudopilus is proposed to access extracellular DNA via the PilQ secretin. Subunits of the major pilin protein, PilA, comprise the pseudopilus, and the IM PilD prepilin peptidase processes the PilA monomers for assembly into the pseudopilin filament. Recent evidence supports that the *Neisseria* minor pilin, ComP, which is dispensable for pilin assembly and yet required for DNA uptake, has characteristics consistent with the DUS receptor (32, 184)**.** Minor pilins required for *Vibrio* competence have not been described, and *V. cholerae* encodes no obvious ortholog of ComP. In Gram$^-$ bacteria, the IM PilC protein is thought to serve as an anchor for pseudopilin assembly. Each naturally competent *Vibrio* encodes a *pilA-D* operon (Table 1.1), and *V.*

cholerae Δ*pilA* and Δ*pilB* mutants have been shown to be defective for DNA uptake (118). Likewise, genome analysis predicts that each competent *Vibrio* also encodes at least two NTPases, PilF and PilT. In other bacteria, PilF energizes pilus assembly, while PilT protein likely promotes disassembly (Fig. 1.1), by analogy to the role of PilT in Type IV pili in *N. gonorrhoeae* (183). Each *Vibrio* is predicted to encode both one PilF and two PilT orthologs annotated as PilT1 and PilU (Table 1.1), although a role for each of these proteins in competence has not yet been confirmed by mutational analysis.

With the exception of *N. gonorrhoeae*, which takes up extracellular DNA constitutively (20), in other well-studied $Gram^-$ and $Gram^+$ bacteria including *B. subtilis*, *S. pneumonia* and *H. influenzae* genes encoding the competence apparatus are only expressed in response to environmental signals and cues (57). In *V. cholerae*, several signal molecules have been identified that trigger DNA uptake, indicating that natural competence is not constitutive. Importantly, data described below supports a model that expression of the *V. cholerae* competence machinery is coordinated by a set of extracellular signals that are apparently exclusive to marine environment and absent in the human host.

1.3. Regulatory Systems

The ability to sense and respond to environmental signals is critical for survival and adaptation by bacteria. In the case of natural competence in *V. cholerae*, much was already understood regarding several well-described signal transduction pathways in *V. cholerae* and related bacteria prior to discovery of their role in DNA uptake. Namely, three distinct signaling pathways converge to regulate the expression of genes required for competence. In each system, the extracellular signal molecules that activate each

pathway have been identified. As in other bacteria, competence in *V. cholerae* is also coordinated in response to starvation. This gives unprecedented opportunity to define the regulatory connections linking these known regulatory systems to the genes required for genetic competence. In particular, *Vibrio* competence is coordinated by a complex array of regulators that couple a starvation response to three extracellular signals: chitin, quorum sensing autoinducer molecules, and extracellular nucleosides.

1.3.1. Chitin utilization system

The most abundant biopolymer in the aquatic environments is chitin, which serves as an important source of organic nitrogen and carbon for marine organisms (2, 117). Chitin is a highly insoluble β -1,4-linked polymer composed of *N*-acetylglucosamine (GlcNAc) and some glucosamine (GlcN) residues (66). Chains of GlcNAc residues are arranged in antiparallel (α) or parallel (β) configurations. Both forms are found in the marine environments: β-chitin is produced by diatoms and is a major component of squid pens, while the α-form makes up crustacean shells. Annual chitin production in aquatic biosphere is estimated to be more than 10^{11} tons, mostly from copepods and other organisms resulting in a continuous 'marine snow' of organic matter down through the water column (157, 191). However, chitin is rapidly recycled by chitinolytic bacteria, including members of the family *Vibrionaceae*, that break down the polymer into its soluble subunits GlcNAc and chitobiose $(GlcNAc)_2$. This chitin utilization process allows chitinolytic bacteria to maintain the biogeochemical cycles of nitrogen and carbon in the marine environments (141).

Vibrio species living in aquatic environments are capable of using chitin as a sole source of carbon. Moreover, it was shown that the chitin degradation pathway is highly

conserved among *Vibrios* suggesting that chitin metabolism is an ancestral function for the genus (92). The connection of *V. cholerae* with chitin is well documented and, for microbial ecology, is one of the best-described examples of a successful bacteria-substrate interaction in the environment (50, 143). Attachment of pathogenic *V. cholerae* to chitinous zooplankton likely provides many benefits to the bacterium by: providing a nutrient-rich habitat (85), enhancing transmission of the bacterium to its human host (49, 94), up-regulating attachment and colonization proteins involved in pathogenesis (102, 148), increasing survival during temperature stress and exposure to stomach acid (3, 127) and by altering bacterial physiology to induce the natural competence program (118). This later role of chitin is our focus here.

1.3.1.1. Binding to chitin

From the perspective of natural transformation in *V. cholerae* and other *Vibrios,* the chitin utilization system is initiated by sensing of chitin and direct binding to chitinous surfaces. Attachment to chitin in the environment may be either a spontaneous transient event or promoted by chitin and chitin oligomers. Li & Roseman have reported chemotaxis of *V. cholerae* toward chitin oligosaccharides (108). Based on previous studies of *V. furnissii*, it is thought that starving cells secrete chitinases that degrade chitin in the microenvironment to generate a gradient of GlcNAc and $(GlcNAc)_2$ (16, 17). Chitin degradation is controlled by multiple chitinases (168), however, for chitinolytic bacteria, *chiA* typically shows the highest expression such that the ChiA enzyme is the most active in response to chitin (135, 168). Bacteria led by the gradient are proposed to swim toward chitinous surfaces and induce several types of adhesive pili promoting colonization (119, 148).

MshA pilin subunits assemble into the Mannose-sensitive haemagglutinin (MSHA) pilus, a type IV pilus that is involved in colonization and biofilm formation by *V. cholerae* on nutritive (cellulose) and non-nutritive (borosilicate glass) surfaces (180). MSHA promotes adherence to both chitin beads (119) and crustaceans or their shells (41, 119). Since crab shells are covered by a non-chitin epicuticle, which must be degraded before chitin degradation can proceed, Meibom *et al* suggested that MSHA might initiate adherence that is independent of surface chemistry (119).

The toxin-coregulated pilus (TCP) is required for intestinal colonization (6, 90, 103, 170) and also serves as the receptor for the cholera toxin-containing CTX phage (177). Though initially believed that it exclusively promotes attachment to human intestinal cells, TCP also appears to participate in biofilm formation on chitin surfaces (148). Using scanning electron microscopy to visualize development of wild-type (WT) and TCP mutant biofilms on the surfaces of chitinous squid pens, it was found that TCP does not function as a bacterial adhesin, but promotes biofilm differentiation on chitinous surfaces by mediating cell-to-cell interactions (148).

GlcNAc binding protein A, GbpA, can also mediate attachment of *V. cholerae* to chitin, GlcNAc oligomers, and exoskeletons of zooplankton, as well as participate in effective intestinal colonization within the infant mouse model of cholera (102, 192). The mechanism by which GbpA mediates attachment remains uncharacterized (102), but the protein possesses an unusual three-dimensional structure consisting of four separate domains; two are similar to domains of proteins known to bind chitin, while the other two are required for cell-cell interactions (185). It is hypothesized that this domain structure creates a stable interface between *V. cholerae* and the human or marine host facilitating

colonization (185). The contribution of MshA, TCP, and GbpA to *V. cholerae* natural competence has not been reported.

Another type IV pilus reported to contribute to colonization of chitin was the PilA-containing chitin regulated pilus (ChiRP) (119). It was initially speculated that a gradient of chitin oligosaccharides produced by chitinolytic bacteria induced synthesis of ChiRP to promote colonization, followed by efficient digestion and assimilation of the polysaccharides (119). However, later Meibom *et al* provided evidence that this type IV-like pilus is instead a component of the competence apparatus for DNA uptake of *V. cholerae* (118).

1.3.1.2. Extracellular chitin as a signal

Recognition of chitin liberated from chitinous surfaces induces a chitin utilization program in *V. cholerae* that is tightly connected to DNA uptake. Secreted chitinases that hydrolyze chitin generate a variety of products including GlcNAc, GlcN, oligosaccharides of both monomers, and oligosaccharides that contain both GlcNAc and GlcN. However, only chitin and chitin oligomers ($(GlcNAc)_n$, n = 2-6) induce the natural competence program (118, 119). Li & Roseman suggested that GlcNAc can be derived from multiple sources in the environment making the monomeric form poorly suited for a signal to cells to start expression of the chitin catabolic machinery. Chitin oligomers, in contrast, are derived exclusively from chitin and, thus, could serve as a specific signal to bacteria that chitin is available (108).

The induction of competence by GlcNAc oligomers is a complex process that consists of multiple steps and involves numerous regulatory and structural proteins (22, 108, 118).

A combination of microarray expression and genetic studies of *V. cholerae* growing on natural chitin surfaces identified a 41-gene regulon involved in chitin colonization, digestion, transport and assimilation, including the genes predicted to encode the competence apparatus (Table 1.1) (118, 119). Meibom *et al* demonstrated that a subset of these genes is under control of the membrane-bound chitin-sensing histidine kinase, termed ChiS (119), consistent with the role of ChiS as a sensor linking chitin to the expression of competence genes.

The expression of the ChiS-dependent chitinolytic and natural competence genes is regulated by a periplasmic chitin oligosaccharide binding protein, CBP (108). Genetic studies suggest that in the absence of chitin, CBP binds to ChiS and inhibits its activity. Based on this model (Fig. 1.2), in the presence of chitin, oligomers such as $(GlcNAc)_2$ activate ChiS by binding to CBP and neutralizing its repressive effects on ChiS (108). A putative cognate response regulator for ChiS remains to be identified. Active ChiS promotes the transcription of a non-coding small RNA (sRNA) called TfoR, presumably via one or more intermediates since ChiS lacks an obvious DNA binding domain. Like many bacterial sRNAs, TfoR requires participation of the RNA binding protein Hfq for recognition of the mRNA target to which it is predicted to bind. Specifically, Yamamota *et al* identified TfoR as a positive regulator of the mRNA that encodes a critical regulator of the competence state, TfoX (187). Thus, TfoR serves as a positive regulator of *tfoX* translation, presumably binding directly to the 5'UTR of *tfoX* and altering secondary structure of the mRNA to promote access of the ribosome to the ribosome binding site (RBS) and to stimulate translation of the TfoX protein (187).

The presence of *tfoX* homologs in all competent *Vibrios* (Table 1.1) combined with the shared capability of *Vibrios* to utilize chitin as a nutrient (92) suggests that chitin-induced

competence may be a shared trait of this genus. However, unlike *V. cholerae,* transformation of *V. fischeri* can be induced only by chitin oligosaccharides but not by chitin in the form of crab shell tiles. It is thought that this defect may be due to the relatively poor attachment and growth of *V. fischeri* on this insoluble substrate (139). Moreover, the transformation rate of *V. fischeri* is ~100-fold lower than in *V. cholerae*, perhaps consistent with differences in competence regulation in this squid symbiont (139), as described below.

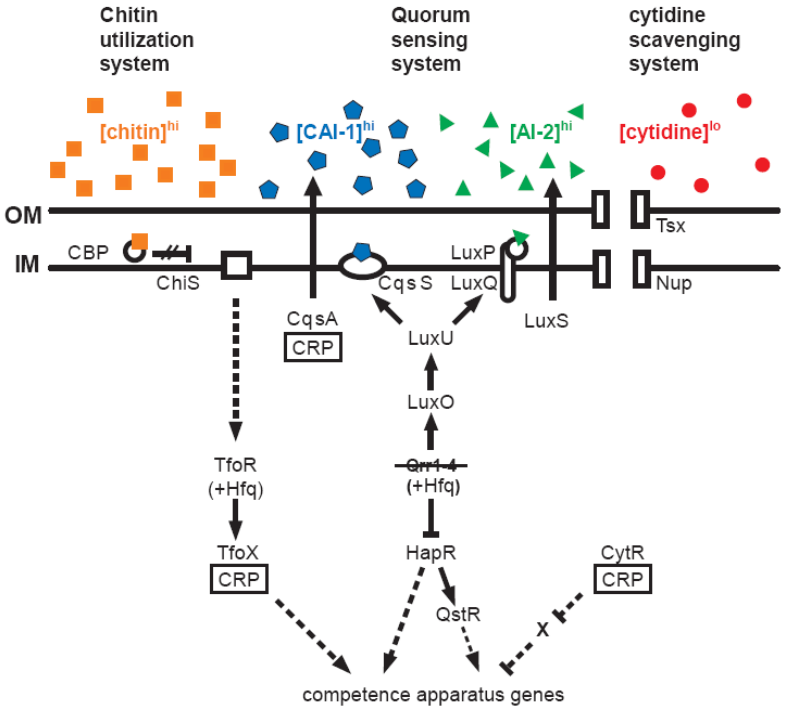

Figure 1.2. A model: multiple regulatory pathways control natural competence in *Vibrio cholerae.* Chitin present at high levels in marine systems, associates with the chitin binding protein (CBP), preventing repression of ChiS and resulting in production of the TfoX regulator. In response to high levels of quorum sensing autoinducers (CAI-1 and AI-2) that bind to cognate inner membrane (IM) receptor proteins, sRNAs (qrr1-4) are not transcribed and the HapR transcription factor is made. HapR directly activates transcription of the QstR regulator. CytR is present to positively control competence when extracellular levels of nucleosides are low and cytidine is not taken up by the Tsx/Nup complex. The catabolite repressor protein (CRP) plays a role in each regulatory system when glucose is scarce: associating with TfoX, and CytR, and enhancing levels of the CAI-2 synthase enzyme CqsA. Dashed arrows indicate where direct interactions are unlikely and additional pathway components remain to be identified (see text for details).

1.3.1.3. TfoX: Mechanism of competence genes regulation, and a role for CRP

TfoX is a critical regulator of DNA uptake in *V. cholerae*, and an ortholog of *H. influenzae* competence regulator, Sxy (118, 119). Induction of TfoX transcription from a non-native promoter is sufficient to induce several chitinases, and a chitoporin, as well as many of the components of the competence apparatus including *pilQ-M*, *pilA-D*, *pilU*, *pilT1*, *pilF*, and *comEA* (Table 1.1) (118, 119). Recently, expression of *comEC* was also shown to be upregulated by TfoX induction (112), supporting a model for TfoX as an important positive regulator of natural competence in *V. cholerae*.

BLAST searches of sequenced *Vibrio* genomes reveal that many of them also contain a putative paralog of *tfoX* designated as *tfoY* (139). TfoX and TfoY have similar protein sequences; and in *V. fischeri* both are apparently required for transformation, as a *V. fischeri tfoX* and a *tfoY* mutant are defective at DNA uptake. Although similar in sequence, each appears to have a unique function because each is incapable of fully compensating for the loss of the other paralog (139). In contrast, a deletion of *tfoY* in *V. cholerae* has no significant effect on transformation ability (Yamamoto S., unpublished). Nonetheless, significant interest remains in *V. cholerae* TfoY, as the 5' UTR of *tfoY* mRNA functions as a riboswitch to posttranscriptionally control its expression in response to binding of the intracellular second messenger cyclic-di-GMP (105, 160, 166). These complex regulatory mechanisms suggest that *tfoX* and *tfoY* have distinct cellular inputs for their activation and promote competence induction under different environmental conditions.

The manner by which TfoX (and TfoY) activates competence gene transcription is not known. In γ-proteobacteria including *E. coli*, *V. cholerae* and *H. influenzae*, TfoX (Sxy) is

thought to promote transcription of competence-related genes by direct interactions with cAMP receptor protein (CRP) (119, 147). CRP is the global regulator of carbon catabolite repression (CCR) in Gram$^-$ bacteria, which together with its allosteric effector cAMP, controls the expression of multiple genes involved in utilization of alternative carbon sources when glucose levels in the cell are low (27, 53). Specifically, *H. influenzae* Sxy (TfoX) is proposed to direct CRP to interact with a competence regulatory element (CRE) sequence (TGCGA-N6-TCGCA) in the *comE1* (*comEA*) and *pilA* promoters, although a precise mechanism has not been described to fully explain how CRP and/or Sxy engage at the CRE site (which is remarkably similar to the CRP binding site: TGTGA-N6-TCACA) (147). Inspection of the promoter region of *V. cholerae comEA* indicates one potential CRE site (TGCGA-N6-AAGCA); and the *pilA* promoter also contains a potential CRE (or CRP) binding site (TGAGA-N6-TCAAA). Thus, it is possible that in *V. cholerae*, as in *H. influenzae,* CRP via TfoX directly promotes transcription of *comEA* and *pilA*. How TfoX activates transcription of *pilQ-M*, *comEC*, and the NTPase genes in *V. cholerae* is not understood.

Several studies support a critical role for CRP in transformation. First, expression of DNA uptake genes in *V. cholerae* is subject to CCR, as exogenous glucose prevents transformation. Second, natural competence is also fully abolished by deletion of the *cyaA* gene encoding the adenylate cyclase enzyme, which catalyzes cAMP production. Finally, a Δ*crp* mutant is not competent for DNA uptake (4, 22, 30, 118, 193). While these studies confirm a role for CRP in transformation, additional reports described below highlight that this global regulator plays multiple roles in control transformation in *V. cholerae*.

Chitin appears to be one of the essential signals for induction of natural competence in *V. cholerae* that is tightly regulated by complex chitin utilization system (118). However, many additional factors (such as the putative cognate response regulator for ChiS), the precise TfoX regulatory mechanism, as well as factors connecting TfoX to each of the competence genes remain unknown. A mechanism that controls transcription of other TfoX-regulated genes predicted by microarray studies also remains to be determined. Finally, natural competence has not been reported in the absence of TfoX induction (either by extracellular chitin or GlcNAc oligomer, or genetically from a constitutive promoter) suggesting that *V. cholerae* will only take up naked DNA under conditions with sufficient chitin or GlcNAc oligomers. It remains possible that other signaling pathways stimulate natural competence in the absence of TfoX induction; however, to date, competence in *Vibrios* is restricted to marine environment where chitin is abundant.

1.3.2. Quorum sensing system

In the last several decades, research by many investigators has revealed that quorum sensing (QS) is a ubiquitous regulatory mechanism used by $Gram^+$ and $Gram^-$ bacteria to incorporate into gene expression the contribution of cell density. QS allows bacteria to regulate gene expression in response to self-produced signal molecules that accumulate as the number of bacteria in a population increase. The *V. harveyi*-like Vibrio QS system elucidated primarily by Bassler and colleagues (see (131) for review) was exploited in 2002 to uncover a similar system in *V. cholerae* (121) described in detail below, followed by the discovery in 2005 that QS is required for natural competence (118). The *V. cholerae* competence regulation has been studied most extensively to date, however, *Vibrio parahaemolyticus*, *Vibrio vulnificus* and *Vibrio fischeri* are all QS-proficient and also capable of taking up extracellular DNA via transformation (40, 70, 139). Features of

each of these *Vibrio* QS system will be noted if differ from *V. cholerae*. Importantly, neither the *V. fischeri* LuxI/R QS system (reviewed in (122)), nor the *V. parahaemolyticus* Scr QS system (reviewed in (59)) that activates transcription of numerous genes including *gbp* (59), have been shown to regulate competence and will not be discussed here.

1.3.2.1. Extracellular autoinducer signals

Quorum sensing is accomplished as a result of the production of extracellular signal molecules called autoinducers (AIs), and the subsequent recognition of and response to these secreted signals. *V. cholerae* produces two autoinducers, CAI-1 and AI-2, which are released from the bacteria into the extracellular milieu (Fig. 1.2). CAI-1, (*S*)-3-hydroxytridecan-4-one, is produced by the CqsA synthase and detected by the cognate membrane-spanning CqsS receptor (132) that behaves as a hybrid histidine kinase (HK). The second AI to which *V. cholerae* responds is AI-2, a furanosyl borate diester, formed by cyclization of the secreted product of the LuxS enzyme, DPD, with boron that is abundant in marine environments (39). The AI-2 receptor LuxP is a periplasmic protein that associates with the HK LuxQ (128, 129). Both *V. cholerae* HKs, CqsS and LuxQ, are capable of donating to or receiving phosphate from the core QS regulatory pathway described below. The core components: LuxU, LuxO, the Qrr small RNA(s), as well as HapR (OpaR/ LitR/ SmcR), are conserved between sequenced isolates of *V. cholerae*, *V. parahaemolyticus*, *V. fischeri*, and *V. vulnificus*, respectively, based on genetic and genomic data.

The LuxS/P/Q system is apparently conserved in all *Vibrios* whereas the CqsA/S system appears to be conserved among many but not all *Vibrios* including *V. cholerae* and *V.*

parahaemolyticus (89, 132). *V. fischeri* lacks CqsA/S and instead codes for the AinS enzyme that produces an acyl homoserine lactone AI (C8), and the HK AinR, the C8 AI receptor. The AinS/R system behaves similarly to CqsA/S in phosphotransfer to the downstream regulator LuxU (123). *V. vulnificus* produces QS AIs (176) and encodes core QS regulatory components, hence it is likely that AI recognition controls expression of these downstream components as in the other *Vibrios*. As a result, members of the genus *Vibrio* differentially regulate expression of genes at low cell density and at high cell density in response to at least two AIs (the general AI-2 and another more specific signal).

1.3.2.2. The QS autoinducer response

When the number of *V. cholerae* cells in a population is low, the extracellular concentration of each AI (CAI-1 and AI-2) is also low. Sensors CqsS and LuxP/Q unbound by their respective ligands operate as kinases that transfer phosphate to the phosphotransfer protein LuxU. LuxU in turn donates phosphate to the response regulator protein LuxO. Phosphorylated LuxO (LuxO~P), in association with the RpoN (σ^{54}) recruits RNA polymerase to the promoters of four non-coding small RNAs (sRNAs) called the Qrrs (Quorum regulatory RNAs). In this way, LuxO, when phosphorylated at LCD, activates transcription of the Qrr sRNAs. The Qrrs are located within intergenic regions on both chromosomes; Qrr1 is located adjacent to the *luxOU* operon on chromosome 1, and the remaining Qrrs are located on chromosome 2 (107). Interestingly, *V. fischeri* only contains Qrr1, while *V. parahaemolyticus* and *V. vulnificus* have five *qrr* genes (107, 123).

V. cholerae encodes numerous sRNAs (11), and to date genetic evidence indicates that the four *V. cholerae* Qrr sRNAs themselves regulate multiple mRNA targets (13, 78, 107,

150, 167). When the Qrrs are produced, the RNA-binding protein Hfq facilitates the binding of Qrr RNA to the 5'UTR of each mRNA target. Of particular importance here is the role of the Qrr sRNAs in regulating HapR, a transcription factor. The site of Qrr binding overlaps the ribosome binding site of *hapR* (13). As a result, Qrr/*hapR* base pairing prevents HapR translation, so that the lack of AIs at LCD results in Qrr transcription and repression of HapR production. Genetic evidence is consistent with the same regulatory mechanism for Qrr control of the HapR homologue in the other *Vibrios*: namely LitR (in *V. fischeri*) (123), and likely in OpaR (in *V. parahaemolyticus*) and SmcR (in *V. vulnificus*) as well.

At high cell density, the *V. cholerae* AIs bind their cognate HK receptors resulting in a conformational change that disrupts kinase activity and reveals the phosphatase activity of each HK (Fig. 1.2). Consequently phosphorylation of LuxO decreases, Qrr levels decrease, and HapR translation proceeds. HapR plays an important role in regulating biofilm formation and several virulence factors important *in vivo* (77, 194, 195), and also promotes expression of the secreted PrtV protease, which protects *V. cholerae* from grazing by predators in aquatic microcosm experiments (174). Importantly, HapR is a required activator of genes necessary for natural transformation (118).

1.3.2.3. Mechanism of HapR regulation of competence genes

Gram$^-$ bacteria *H. influenzae* and *N. menigitidis* rely on DUS sequences to restrict uptake to species specific DNA and do not regulate competence via QS. In contrast, Gram$^+$ *B. subtilis* and *S. pneumoniae*, like *V. cholerae*, induce competence genes in response to accumulated AI molecules. However, Gram$^+$ QS system utilizes distinct peptide-based QS systems unrelated to the system used by *V. cholerae* and other

Gram$^-$ *Vibrios* (171). Nonetheless, the role of QS as a positive regulator in *V. cholerae* like in other bacteria has been proposed to provide a mechanism to increase the likelihood of taking up extracellular DNA from self or related *Vibrios* (5, 165).

HapR functions in many ways to regulate genes important for natural competence. One important HapR-controlled gene is *dns*, encoding the periplasmic extracellular nuclease, which processes dsDNA allowing for efficient transport of ssDNA through the IM. As discussed, the DNase enzyme was shown to be the primary nuclease required for efficient uptake, as Δ*dns* strains are altered in DNA uptake. HapR is a repressor of *dns*, and Δ*hapR* mutants that are de-repressed for *dns* are not competent. By contrast, Δ*dns* mutants are hypercompetent, presumably because Dns is no longer repressed at high cell density, and deletion of *dns* in a Δ*hapR* mutant partially restores DNA uptake (23). Although a degenerate HapR binding motif has been identified in several promoters shown to be directly bound by HapR (172), the *dns* promoter appears to lack such a motif. Nonetheless, Lo Scrudato *et al* recently demonstrated binding of purified HapR to *dns* promoter DNA supporting a model that HapR may indeed represses *dns* transcription by direct interaction (113).

The contribution of HapR to competence is not restricted to its role in *dns* expression. Strains of *V. cholerae* with Δ*hapR* deletions, which are not competent for DNA uptake, are also severely impaired for transcription of *comEA* (5, 118, 165), and also modestly impaired for transcription of *comEC*, which encodes the IM channel (112). Most recently, Lo Scrudato *et al* have identified the QstR regulator in *V. cholerae* A1552 strain, which provides a positive link between HapR and these competence genes (113). Namely, HapR binds directly to the promoter of QstR, annotated as a LuxR-type response regulator, and a *V. cholerae* Δ*qstR* mutant was reduced for expression of *comEA* and

comEC. It remains unclear how QstR regulates these two competence genes, as QstR was not shown to have DNA binding activity (113). BLAST analysis indicates that *V. vulnificus* and *V. parahaemolyticus* encode a *qstR* ortholog, however, *V. fisheri* lacks this regulator (Table 1.1). Importantly, one or more intermediate factors likely connect QstR to *comEC* and *comEA* transcription.

It has been reported that, like *comEA* and *comEC*, *pilA* transcription is also positively controlled by HapR in strain C6706 (4), although such regulation has not been observed in strain A1552 (112, 113). Specifically, *pilA* expression was reduced in a C6706 Δ*hapR* mutant, similar to results observed for *comEA* expression in this strain. It has been proposed that strain differences may explain this discrepancy, and it is unknown whether a C6706 Δ*qstR* mutant behaves in a manner similar to a A1552 Δ*qstR* mutant. Studies showing that HapR directly regulates numerous genes both positively and negatively by direct promoter binding, indirectly controls expression of several other targets via modulation of c-di-GMP levels (131), and in particular controls several genes involved specifically in natural competence, confirm that HapR's control over DNA uptake is likely to be complex.

Finally, as for TfoX, CRP also appears to contribute to the QS pathway and thus impinge on natural competence. Liang, *et al* demonstrated in El Tor strain C7258 that Δ*crp* mutants are reduced for transcription of the major autoinducer synthase gene *cqsA* (109). Since both A1552 and C6706 Δ*cqsA* mutants are defective in DNA uptake (4, 22), one effect of CRP on competence likely also includes modulation of QS (Fig. 1.2). As such, it is proposed that in response to CCR, *cqsA* transcription is elevated and CAI-1 levels are increased, promoting production of HapR.

1.3.3. Nucleoside transport

When starved for preferred nutrients, many bacteria scavenge from the environment low concentrations of nucleosides that serve as a source of nitrogen and carbon for growth and as precursors for nucleic acid synthesis (1, 81, 126, 130, 133). Evidence that *H. influenzae* competence is inhibited by extracellular nucleosides led to the hypothesis that DNA uptake can not only promote HGT but also serve as mechanism for acquiring nucleic acid precursors (115). By this hypothesis, a depletion of nucleoside pools signals DNA uptake, while an excess of nucleosides halt competence. In *V. cholerae*, recent evidence linking nucleoside uptake to competence bolsters such an argument and is described below (4).

In *E. coli*, passive transport of nucleosides across the OM into the periplasm is mediated by the Tsx porin, while active transport across the IM into the cytoplasm is facilitated by Nup (nucleoside uptake) permeases (Fig. 1.2) (51, 125, 181). In *E. coli*, NupC and NupG are the predominant routes for nucleoside uptake; and Δ*nupC* and Δ*nupG* mutants are incapable of high-affinity nucleoside uptake or growth on nucleosides as single carbon source (126). NupG can transport a wide range of nucleosides and deoxynucleosides while NupC does not transport guanosine or deoxyguanosine.

V. cholerae encodes three homologs of NupC family proteins (VC1953, VC2352, VCA0179), and an ortholog of the Tsx porin, which is annotated as OmpK (VC2305). Although the crystal structure of a *V. cholerae* NupC homolog (VC2352) has recently been determined (98) and the crystal structure of *E. coli* Tsx was solved (189), much remains to be learned about the mechanisms by which these transporters recognize and deliver selective nucleosides. In *E. coli*, transcription of *tsx* and *nupC* is under control of a nucleoside scavenging regulator, CytR, the Cytidine Repressor protein.

1.3.3.1 CytR: CRP-dependent anti-activation of DNA uptake

Recently, Antonova *et al* demonstrated a regulatory link between a nucleoside scavenging system in *V. cholerae* and natural competence (4), as described prior for *H. influenzae* as well (115). Specifically, in a genetic screen for *V. cholerae* mutants defective in *comEA* transcription, Antonova *et al* identified CytR as a positive regulator of competence gene expression and DNA uptake. *V. cholerae* Δ*cytR* mutants are severely impaired for transcription of *comEA*, *pilA* and also for the chitinase gene *chiA-1*. The manner in which CytR positively controls *V. cholerae* competence is currently being defined, but genetic evidence suggests that the molecular mechanism of CytR control is similar to that in *E. coli*.

When free nucleosides are scarce in *E. coli*, the set of nucleoside transport (*nupG* and *tsx*) and metabolism genes (*udp*, *cdd*, *deoC*) is not activated (for review see (175)). Repression (termed “anti-activation” as described here) of this set of genes is orchestrated by interplay between the CytR protein and CRP (see (28) for review). Each promoter contains a proximal and distal binding site for a CRP dimer, separated by ~52 nucleotides. CytR binds via protein-protein interactions with each CRP dimer to a degenerate DNA sequence between the proximal and distal CRP binding sites. In this manner, each promoter is “anti-activated” by CytR. However, in *E. coli*, when free cytidine nucleoside is abundant, binding of the nucleoside to CytR alters the conformation of CytR protein preventing its interaction with CRP protein at each promoter (in a manner analogous to the derepression of the *lac* promoter by binding of allolactose to the LacI repressor). In the absence of CytR, each CRP dimer recruits an α-subunit C-terminal domain of RNA polymerase thereby promoting CRP-dependent activation of nucleoside scavenging genes, such as *udp*, which encodes uridine

dephosyphorylase. As in *E. coli*, CytR-CRP interactions in *V. cholerae* also anti-activate *udp* transcription, as Δ*cytR* mutant shows elevated levels of *udp* transcription (83), and amino acid substitutions in CytR preventing its association with CRP mimic that of a Δ*cytR* mutant (4).

While in *E. coli* CytR anti-activates (or represses) genes, in *V. cholerae* genetic evidence supports a model that CytR anti-activation has a positive effect on natural competence. Namely, *V. cholerae* Δ*cytR* mutants are defective for competence gene expression (*comEA* and *pilA*) and DNA uptake, and addition of exogenous cytidine results in similar effects. While it remains possible that *V. cholerae* CytR may not act in an analogous manner to its *E. coli* counterpart as an anti-activator, Antonova *et al* favor a speculative model that CytR-CRP anti-activates (represses), the promoter of a putative factor X, which in turn behaves as a negative regulator of competence genes (Fig. 1.2). These results are consistent with observations that *comEA* is expressed maximally in wild type *V. cholerae* ($CytR^+$, CRP^+), minimally in Δ*cytR* mutants ($CytR^-$, CRP^+), and at intermediate levels in a Δ*cytR*, Δ*crp* double mutant ($CytR^-$, CRP^-) where *comEA* is neither activated nor anti-activated (4).

Discovery of the promoters directly under CytR-CRP control in *V. cholerae* will provide additional insight into how CytR-CRP mediates its effects on competence and help identify putative factor X (Fig. 1.2). In addition to *udp*, genomic analysis indicates that *tsx* (*ompK*) and *cdd* may also be direct targets of CytR, containing two CRP binding sites separated by 52 nucleotides. However, none of the three NupC-like proteins annotated in the *V. cholerae* genome, appears to carry the two CRP binding sites observed in *E. coli nupC* promoter region (156) (Antonova & Hammer, unpublished).

Previously, it was demonstrated that CytR represses biofilm formation in *V. cholerae* strain MO10, although a direct mechanism linking CytR to biofilm genes was not revealed (83). Recently, Garavaglia *et al* have also demonstrated CytR repression of *E. coli* biofilms, as a ΔcytR mutant displayed reduced expression of the *csgDEFG* operon, which controls assembly and transport of curli fibers that promote aggregation (65). Modulation of intracellular pyrimidine concentrations appears responsible for the changes in curli expression leading the authors to propose that biofilm gene expression is an indirect consequence of CytR control of nucleoside pools in the cell. Thus, it remains possible that CytR does not function via a putative X, but rather indirectly to control competence and biofilms in *V. cholerae*.

A cytidine-responsive CytR-dependent nucleoside scavenging mechanism, described in *E. coli* (10), seems to be a critical component of a regulatory network coupling nutrient stress to natural transformation in *V. cholerae* (4) as described in *H. influenzae* (115). However, the precise CytR regulatory mechanism as well as putative components (such as X) has remained unknown. The fact that CytR anti-activation requires CRP reinforces the critical role for CRP in *V. cholerae* natural competence (Fig. 1.2). Since CRP is a pleiotropic regulator in *E. coli*, it is not surprising that CRP impinges on each of the three major regulatory inputs that control DNA uptake in *V. cholerae*. The contribution of this CCR response and nucleoside scavenging also provides evidence for the function of DNA uptake in *V. cholerae* as described below.

1.4. Function(s) of DNA uptake in *V. cholerae*

"Why" bacteria such as *V. cholerae* become naturally competent to take up extracellular DNA is a question that has persisted since the pioneering studies of the "transforming

principle" by Griffith and later Avery, McLeod and McCarthy who established *S. pneumoniae* as a model organism for studying natural competence for DNA uptake (7, 67). As DNA uptake was later uncovered in diverse bacterial species, hypotheses developed that natural competence evolved to aid in three major processes: HGT, nutrition, and DNA repair (for reviews see (57, 146, 163)). It is acknowledged that DNA taken up by bacteria may not be used exclusively for one function or another, since extracellular DNA scavenged as nutrient may also be available for recombination onto the chromosome and for repair of damaged DNA when of sufficient sequence identity (47, 57, 124). Current studies of natural competence suggest that DNA taken up by the competence apparatus may indeed serve multiple functions in the marine pathogen *Vibrio cholerae* as well.

1.4.1. HGT

Genome sequencing efforts for many bacteria, including *Vibrios*, have revealed that horizontal acquisition of DNA by conjugation, transduction and transformation has been responsible for diverse adaptation within microbial genomes (43, 134). Virulence of *V. cholerae* is likely the result of multiple HGT events that allowed a benign marine bacterium to evolve into a major human pathogen (149). Indeed, the essential virulence factor, cholera toxin, was shown in 1996 to be encoded on a bacterial prophage integrated into the *V. cholerae* genome (177)**.** The O139 serogroup of *V. cholerae* that emerged on the Indian subcontinent in 1992 also evolved from an O1 El Tor ancestor through horizontal acquisition of the genes encoding for the O139 antigen and capsule (19). Chitin-induced natural competence has since been shown to be a mechanism that could promote both successful serogroup conversion of an O1 recipient by an O139 donor, and the transfer of cholera toxin genes from one serotype to another (24, 173).

The presence of extracellular DNA in *V. cholerae* biofilms confirms that, like other bacteria, this marine bacterium contributes to a pool of extracellular genetic material that may aid in the evolution of *Vibrios* by natural competence (155).

Once extracellular DNA is transported to the cytoplasm of *V. cholerae* by the competence apparatus, successful recombination depends on the extent of DNA sequence similarity between recipient genome and incoming DNA (116). Thus far, chitin-induced horizontal exchange of genetic material has been documented in lab settings between different *V. cholerae* isolates, but not between different members of the *Vibrio* genus (24, 118, 173). Co-occurrence of *Vibrios* has been documented in environmental settings (52, 82, 100), thus opportunities likely exist for cross-species DNA exchange. Sequence divergence between species members likely prevents extensive HGT or makes these rare events difficult to document by current methods for recording HGT in *Vibrios*. In such cases, liberation of nucleic acids by periplasmic nucleases may serve an additional function in *Vibrios*, namely food.

1.4.2. Nutrition

"Nutritional competence" as opposed to natural transformation describes the processes by which exogenous DNA taken up by the competence machinery serves as a nutrient source for starving bacteria because the ability to "eat" DNA provides a competitive advantage in nutrient-limited environments (136). The "DNA for food" hypothesis is strongly supported by numerous studies in many $Gram^-$ and $Gram^+$ bacteria (35, 57, 60, 146). For example, *E. coli* can utilize extracellular DNA as a sole source of carbon and energy; however, mutants deleted for genes homologous to *H. influenzae* competence genes are unable to do so (60).

Initially the role of DNA as a nutrient source for naturally competent bacteria was proposed by Redfield for *H. influenzae* and *B. subtilis* (146), following the 1992 study by Chandler demonstrating that *H. influenzae* competence is under control of CCR that serve to measure energy insufficiency (33). Later, MacFayden *et al* demonstrated that the presence of extracellular purine ribonucleotides also represses transcription of competence genes and competence development *H. influenzae* (115). It was noted that *H. influenzae* lacks CytR, the pyrimidine scavenging regulator, but encoded the purine regulator, PurR. However *H. influenzae* mutants defective for PurR have not been described. *V. cholerae* natural competence is likewise regulated by a TfoX-dependent CCR response, and a CCR-dependent scavenging system, controlled by CytR (Fig. 2) (4, 22).

Studies by Seper *et al* are consistent with a model that the role of naked DNA extends beyond HGT, since *V. cholerae* is capable of utilizing extracellular DNA as a phosphate source due to the activity of the secreted Dns extracellular nuclease that is under QS control (155). Because *V. cholerae* transits between two distinct environments along its life cycle, it remains possible that this microbe evolved systems to utilize exogenous DNA as a source of nutrients. As proposed for *H. influenza* (115), perhaps an abundance of nucleosides available from neighbor cell lysis triggers a halt in production of the competence apparatus in *V. cholerae* in favor of plentiful extracellular nucleic acid pools.

1.4.3. Repair

The main support of "DNA for repair" is based on observations that several naturally competent bacteria have mechanisms to discriminate between self and foreign DNA. As discussed, QS does not control natural competence in *N. gonorrhoeae* and *H. influenzae*, which only take up DNA carrying DUS, perhaps to ensure "sexual isolation" (36). In contrast, Gram$^+$ *S. pneumoniae* and *B. subtilis* require a peptide-based QS system to take up DNA without sequence specificity, perhaps limiting competence induction to conditions that may favor acquisition of "self" but not "foreign" DNA (47, 57, 171). QS-proficient *V. cholerae* lacks uptake sequences and instead takes up DNA broadly (165), in contrast to the archetypal Gram$^-$ *N. gonorrhoeae* and *H. influenzae*. Poor sequence identity apparently prevents incorporation by *V. cholerae* of DNA from distantly related bacteria, because transformants are detected only in experiments where the donor DNA originates from other *V. cholerae* isolates (24, 118, 173) but not from other *Vibrio* species, or other bacteria such as *E. coli* (165) (Antonova & Hammer, unpublished results).

It is typically believed that transformation involves the uptake of DNA passively released into the environment as a consequence of bacterial growth and senescence (35). However, competent *S. pneumoniae* cells release factors to induce killing of non-competent siblings in a population (45), and *N. gonorrhoeae* also invoke DNA release from neighboring cells (74). Although a general stress response induces competence in both *B. subtilis* and *S. pneumoniae* (47), DNA damage has not been shown to promote transformation as part of an SOS response to repair or bypass DNA lesions. The Gram$^-$ human stomach pathogen, *Helicobacter pylori*, which is likely exposed to constant DNA damage and requires DNA repair systems to survive in the host, lacks an SOS response like many bacteria including *V. cholerae*. Nonetheless, *H. pylori* is proposed to use DNA

uptake for repair as it induces both DNA uptake machinery and enzymes to liberate DNA from neighboring cells (55). *Legionella pneumophila,* which also lacks an SOS response, in a similar manner induces competence for natural transformation as a response to antibiotics and UV radiation (34).

V. cholerae indeed mounts an SOS response when exposed to DNA damaging agents and recent studies suggest a potential link between SOS-induction and competence. Specifically, conditions that induce an SOS response can promote transcription of the integrase enzyme that mediates recombination events at the superintegron on *V. cholerae* chromosome 2 (69). Since ssDNA entering the *V. cholerae* cytoplasm by the competence apparatus may also induce the SOS response (8), future studies may indeed connect transformation to an SOS-controlled DNA repair.

1.5. Conclusion

Multiple signaling systems coordinate DNA uptake in *V. cholerae*. However, many regulatory details remain unknown that directly connect each signaling system to the genes encoding the competence apparatus. Based on studies with limited *Vibrio* strains tested to date, competence is closely linked to a set of signals specific to marine settings. Future studies with these and other isolates will identify additional components and perhaps novel regulatory inputs that turn “on” and “off” DNA uptake in the *Vibrios*. Thus far, only strains with defects in the QS pathway have been described that are also defective for natural competence. However, further studies of both environmental and pathogenic *Vibrios* may identify isolates with novel defects that compromise HGT via the competence apparatus. Such studies will continue to define the role of natural competence in genome evolution and fitness of the *Vibrio* bacteria.

CHAPTER 2

QUORUM SENSING AUTOINDUCER MOLECULES PRODUCED BY MEMBERS OF A MULTI-SPECIES BIOFILM PROMOTE HORIZONTAL GENE TRANSFER TO *VIBRIO CHOLERAE*

2.1. Abstract

Vibrio cholerae, the causative agent of cholera and a natural inhabitant of aquatic environments, regulates numerous behaviors using a quorum sensing (QS) system conserved among many members of the marine genus *Vibrio*. The *Vibrio* QS response is mediated by two extracellular autoinducer (AI) molecules; CAI-I, which is only produced by Vibrios, and AI-2, which is made by many bacteria. In marine biofilms on chitinous surfaces, QS-proficient *V. cholerae* become naturally competent to take up extracellular DNA. Because the direct role of AIs in this environmental behavior had not been determined, we sought to define the contribution of CAI-1 and AI-2 in controlling transcription of the competence gene, *comEA,* and in DNA uptake. In this study we demonstrated that *comEA* transcription and the horizontal acquisition of DNA by *V. cholerae* are induced in response to purified CAI-1 and AI-2, and also by AIs derived from other *Vibrios* co-cultured with *V. cholerae* within a mixed-species biofilm. These results suggest that AI communication within a consortium may promote DNA exchange among Vibrios, perhaps contributing to the evolution of these bacterial pathogens.

2.2. Introduction

Vibrio cholerae, a common marine bacterium and the causative agent of the disease cholera, produces and then responds to extracellular small molecules called

autoinducers (AIs) to collectively control gene expression and coordinate group behaviors, a process called quorum sensing (QS) (64, 131). Specifically, *V. cholerae* produces two AIs; CAI-I (the product of the CqsA synthase), which is restricted to *Vibrios*, and AI-2 (the product of the LuxS synthase), an inter-species AI molecule produced by many bacteria (39, 91, 186). At low cell density (low AI levels) the phosphorylated response regulator LuxO activates transcription of multiple small RNAs that base-pair with and alter translation of several other mRNAs; most notably repressing translation of *hapR*, which encodes the master regulator of QS (78, 107, 150, 167). At high cell density (high AI levels), the binding of AIs to their cognate receptors results in dephosphorylation and inactivation of LuxO, so that HapR is made. HapR represses multiple genes, and also activates others, such as the gene coding for ComEA, a ssDNA binding protein required for DNA uptake or horizontal gene transfer (118) (Fig. 2.1). Thus, wild type *V. cholerae* strains are naturally competent at high cell density, a Δ*hapR* mutant does not take up DNA, and a Δ*luxO* strain that constitutively expresses HapR is capable of *comEA*-dependent DNA uptake even at low cell density (23, 118). A *V. cholerae*-like QS pathway is well conserved in other *Vibrio* species, such as *Vibrio harveyi*, which also produces both CAI-1 and AI-2 (79).

Vibrios commonly form biofilms in marine environments on abiotic and biotic surfaces and it was recently shown that QS-dependent DNA uptake by *V. cholerae* requires the presence of chitin, such as found in copepods molts and crab shells (95, 100, 118). A chitin responsive pathway induces transcription of several genes including *tfoX* that encodes an additional regulator required along with HapR for postive control of *comEA* transcription (105, 160, 188) (Fig. 2.1). *Vibrio* species can often be found together in marine settings (52, 95, 100, 101, 114, 161) and can communicate with each other using both CAI-1 and AI-2 (18, 89, 131). In this study we tested the hypothesis that AI

molecules made by different bacteria within a mixed-species biofilm induce horizontal gene transfer (HGT) to *V. cholerae* (14).

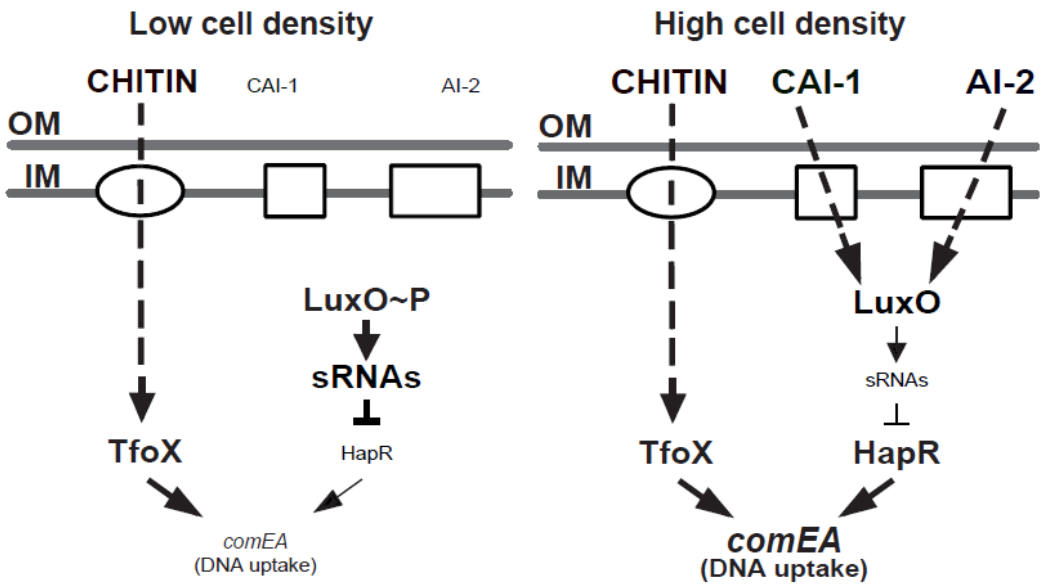

Figure 2.1. Activation of *comEA* transcription and natural competence in *V. cholerae* by quorum sensing autoinducers in the presence of chitin. At low cell density (low CAI-1 and AI-2 levels), when HapR is repressed, *comEA* expression is insufficient to promote DNA uptake even in the presence of TfoX, which is induced by chitin (left panel). At high cell density, the quorum sensing AI response results in production of HapR, which along with chitin-induced activation of TfoX, promotes sufficient transcription of *comEA* to mediate natural competence for DNA uptake (right panel). Refer to the text and (118, 131) for details of signal transduction pathways depicted as dashed lines). OM, outer membrane; IM, inner membrane.

2.3. Materials and methods

2.3.1 Bacterial strains, plasmids, and culture conditions

The relevant genotypes of the *Vibrios* strains, and plasmids used in the study are listed in Table 2.1. *V. cholerae* and *V. parahaemolyticus* strains were incubated at 37°C on Luria-Bertani (LB) agar, and in LB broth with shaking. In co-culture experiments with *V. harveyi* and *V. fischeri,* the *Vibrios* were incubated at 30°C and 28°C, respectively, and the AI donors were incubated on Luria-Marine (LM) agar for quantification, and in LM

broth prior to co-culturing. The antibiotics (Fisher BioReagents) chloramphenicol (Cm), kanamycin (Kan), and streptomycin (Str) were used at concentrations of 10, 100, 5000 µg/ml, respectively. Expression of the *tfoX* gene encoded on p*tfoX* was induced with 0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG; Fisher BioReagents).

Table 2.1. Bacterial strains and plasmids used in this study

Strains	Genotype or description	Reference
V. cholerae strains		
C6706str	El Tor biotype, O1; $HapR^+$	(170)
BH1515	Δ*luxS*	This study
BH1523	Δ*cqsA*	This study
BH1575	Δ*cqsA* Δ*luxS*	This study
SLS349	Δ*luxO*	(179)
BH1543	Δ*hapR*	This study
EA093	Δ*luxS* Δ*hapR*	This study
EA094	Δ*cqsA* Δ*hapR*	This study
BH2104	Δ*cqsA* Δ*luxS* Δ*hapR*	This study
EA090	Δ*lacZ*::Kan^R	This study
V. harveyi strains		
BB120	ATCC BAA-1116	(18)
KM387	Δ*luxS*	(89)
JMH603	Δ*cqsA*	(89)
JMH606	Δ*cqsA* Δ*luxS*	(89)
Other *Vibrio* strains		
LM4437	*V. parahaemolyticus*, Δ*opaR*::Tn*5*	(88)
ES114	*V. fischeri*, Wild Type	(25)

Plasmids	Features	Reference
pEVS143	Cloning vector, Kan^R	(58)
pBBRlux	Cloning vector, Cm^R	(107)
pEA201	p*tfoX* , pEVS143-based, Kan^R	This study
pEA209	p*comEA-lux*, pBBRlux-based, Cm^R	This study
pEA089	pKAS-Δ*lacZ*::Kan^R	This study

2.3.2. DNA manipulations

Standard protocols were used for all DNA manipulations (151). Restriction enzymes, T4 DNA ligase (New England Biolabs), and Phusion DNA polymerase (Finnzymes) were

used for cloning and PCR reactions. Standard methods were used to make deletion constructs (159), as well as the Kan^R *V. cholerae* strain, which contained a copy of the Kan^R cassette from plasmid pEVS143 integrated at the *lacZ* site (58). Genomic DNA from the *V. cholerae* Δ*lacZ*::Kan^R strain was extracted using a ZR Fungal/Bacterial DNA kit™ (Zymo Research) for experiments measuring the uptake of DNA. The luciferase-based reporter plasmid, p*comEA-lux*, was constructed by PCR amplifying the promoter and a portion of the coding region of *vc1917* from WT *V. cholerae*, and then cloning it into the pBBRlux vector (described in (107)) by insertion into the SpeI and BamHI restriction sites. The IPTG-inducible p*tfoX* plasmid was constructed by amplifying the entire coding region of *vc1153* and cloning it into the pEVS143 vector by insertion into the EcoRI and BamHI restriction sites.

2.3.3. Bioluminescence assay

Plasmid p*tfoX* was introduced by conjugation into *V. cholerae* strains carrying p*comEA-lux*. For measurement of *comEA-lux* expression, *V. cholerae* strains carrying both plasmids were grown in LB with appropriate antibiotics at 37°C overnight, diluted 1:1000 into fresh medium, and incubated for approximately 8 h. To measure *comEA-lux* expression in response to purified AIs, the *V. cholerae* AI-deficient recipient was incubated as described above, but diluted 1:1000 into fresh medium containing purified CAI-1 alone, AI-2 alone, or both AIs at a final concentration of 10 μM, and incubated for 8 h. Purified AIs were prepared as described (91, 152). Bioluminescence was measured using a Wallac model 1409 liquid scintillation counter as described previously (78). Relative Light Units (RLU) are defined as counts per min^{-1} ml^{-1}/OD_{600}. Single-time-point experiments were performed with triplicate samples.

2.3.4. Chitin-induced natural transformation assay

Chitin-induced transformation experiments were performed as described previously (118). In transformation experiments with purified AIs, crab shells were inoculated with 2 ml of the *V. cholerae* AI-deficient strain, and supplemented with purified AIs (each at 10 μM concentration) at the time of inoculation of the crab shells and again 24 h later along with 2 μg of genomic DNA marked with the Kan^R gene. In mixed-species transformation assays, crab shells were inoculated with the *V. cholerae* AI-deficient recipient and the *Vibrio* AI-donor at a 1:1 ratio and incubated for 24 h. After addition of marked genomic DNA, biofilms were grown for an additional 24 h before harvesting and plating to determine transformation efficiency defined as Kan^R CFU ml^{-1}/total CFU ml^{-1} (as described previously in (118)). In all mixed-species experiments, harvested cells were plated onto selective media to determine the total number of CFU and the number of transformants. *V. cholerae* was selected on LB containing Str. The *V. cholerae* AI donor strains (BH1543, EA093, EA094 and BH2104) used in the control co-culture experiments were $HapR^-$ (and thus QS^-), and displayed a rugose colony morphology easily distinguishable from the *V. cholerae* AI-recipient (77). No Kan^R $HapR^-$ (rugose) colonies were detected in these transformation experiments. Since the *V. harveyi*, *V. fischeri*, and *V. parahaemolyticus* strains used are Amp^R (and also Str^S), these strains were selected on LM and LB containing Amp, respectively. For enumeration of transformants, cultures were plated onto LB medium containing Kan and Str. Independent experiments were performed in triplicate.

2.4. Results

2.4.1. AI-deficient mutants of *V. cholerae* are impaired in expression of the *comEA* gene and in DNA uptake

Previous studies with *V. cholerae* mutants (Δ*hapR* and Δ*luxO*) documented that in addition to the chitin controlled TfoX pathway, quorum sensing (QS) is required for the activation of *comEA* transcription (23, 118) (Fig. 2.1). We introduced into *V. cholerae* strains a plasmid-borne transcriptional reporter gene fusion of *comEA* to the luciferase operon (p*comEA-lux*), and an inducible *tfoX* plasmid (p*tfoX*) that alleviated the need for chitin in experiments monitoring *comEA* expression. As described previously, both wild-type (WT) *V. cholerae* and a Δ*luxO* mutant express *comEA*, while a Δ*hapR* mutant is ~100-fold reduced in *comEA* expression (Fig. 2.2A). To define the role of AI molecules in the regulation of the *comEA* gene, we next measured the expression of p*comEA-lux* in *V. cholerae* mutants that produce only CAI-1 (Δ*luxS*), only AI-2 (Δ*cqsA*), or neither AI (Δ*cqsA* Δ*luxS*). The Δ*luxS* strain producing CAI-1 expressed p*comEA-lux* at levels less than WT, expression was further reduced for the Δ*cqsA* mutant that only produces AI-2, and the AI-deficient mutant (Δ*cqsA* Δ*luxS*) expressed *comEA* at levels similar to the QS-deficient Δ*hapR* mutant (Fig. 2.1). As expected, a Δ*tfoX* strain only activates *comEA* expression when induced to express TfoX from the plasmid; and the absence of TfoX induction reduced *comEA* expression in all strains to levels ~100 lower than the Δ*hapR* mutant (Fig. 2.2A, white bars). Thus, TfoX is required for *comEA* transcription, and CqsA and LuxS together enhance expression ~ 50-fold relative to the Δ*hapR* mutant. CAI-1 is the major AI, and AI-2 is the minor AI for *comEA* transcription, as reported for *V. cholerae* virulence factor production *in vivo* (56, 91).

To quantify the contribution to DNA uptake of AIs produced by *V. cholerae*, we measured transformation frequency of *V. cholerae* WT, Δ*hapR*, and Δ*luxO* strains using a crab-shell microcosm system described previously (118). Transformation efficiency of WT and the Δ*luxO* mutant were maximal, and no transformants were detected with the Δ*hapR* mutant (Fig. 2.2B). The Δ*luxS* mutant, which produces CAI-1, was approximately 4-fold impaired for transformation, however, both QS mutants (Δ*cqsA* and Δ*cqsA* Δ*luxS*) that do not make CAI-1 were severely compromised for transformation ~100-fold relative to WT (Fig. 2.2B). No transformants were obtained in the absence of extracellular Kan^R DNA, or when extracellular DNA was unmarked (data not shown), and in Δ*tfoX* (Fig. 2.2B), and Δ*comEA* mutants, as described previously (118). Thus, AIs produced by *V. cholerae* within a single-species biofilm promote DNA uptake. The discrepancy between the transformation frequency of the Δ*cqsA* Δ*luxS* and the Δ*hapR* mutants may reflect that QS sRNAs constitutively expressed in the AI-deficient strain do not completely eliminate all *hapR* mRNA (12). Apparently low levels of HapR protein can occasionally promote DNA uptake in this 24 h assay where rare transformation events may be amplified by replication. Alternatively, it is possible that the presence of chitin used for transformation measurements (Fig. 2.2B) may provide additional signaling information that is absent when *comEA* expression is measured in the presence of TfoX induction rather than chitin (Fig. 2.2A).

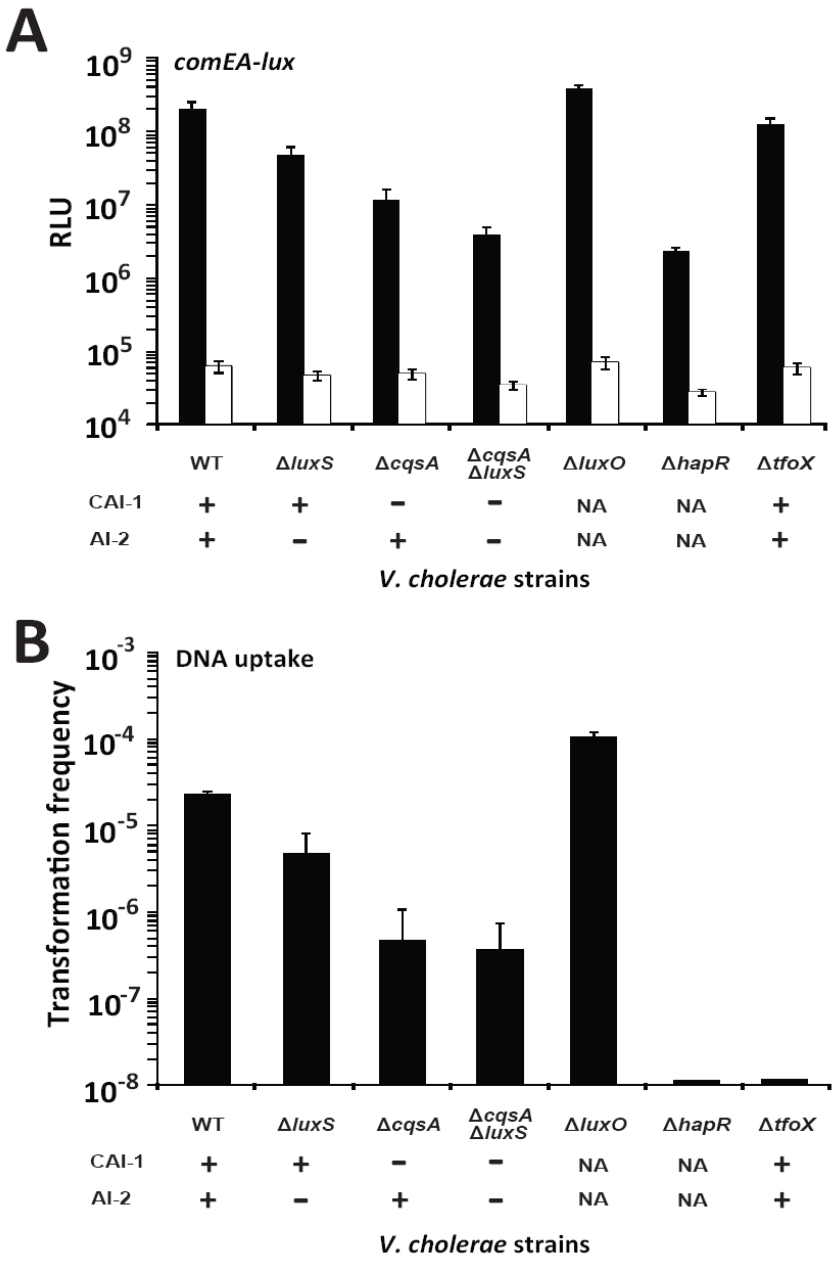

Figure 2.2. Expression of p*comEA-lux* and DNA uptake are controlled by *V. cholerae* autoinducers. (A) *V. cholerae* strains carrying a p*comEA-lux* plasmid and an IPTG-inducible *tfoX* plasmid were incubated with (black bars) or without IPTG (white bars). Bioluminescence is defined as relative light production per OD_{600} (RLU). Triplicate cultures were incubated overnight and analyzed for light. **(B)** Chitin-induced transformation frequency was calculated (see Material and methods) for each *V. cholerae* strain incubated with extracellular DNA in triplicate wells carrying crab shell fragments. Indicated below figures are AIs made by each strain, and NA denotes that AI production by the QS "locked" mutants is not applicable. The limit of detection of the experiment presented was 1.0×10^{-8}. Data shown are mean values ± standard deviation from one representative experiment of three performed.

2.4.2. Purified AI molecules activate the *comEA* gene and DNA uptake by *V. cholerae*

To test directly the role of AIs in *comEA* transcription and DNA uptake, purified CAI-1 and AI-2 where applied to the *V. cholerae* AI-deficient Δ*cqsA* Δ*luxS* mutant under the conditions described above. As shown for the *V. cholerae* AI synthase mutants (Fig. 2.2A), the presence of both purified AIs (at saturating concentrations of 10 μM) resulted in maximal *comEA* expression by the AI-deficient *V. cholerae* strain, and slightly lower levels were obtained when purified CAI-1 was provided alone (Fig. 2.3A). Expression was reduced further when only AI-2 was provided, and the lowest *comEA* transcription was observed when neither AI was provided (Fig. 2.3A). Likewise, a similar pattern was observed with the purified AIs in the crab-shell microcosm assay with the AI-deficient *V. cholerae* Δ*cqsA* Δ*luxS* mutant. We suspect that the slightly lower levels of *comEA* expression observed when the AIs were produced by *V. cholerae* (Fig. 2.2A) compared to the results with purified AIs (Fig. 2.3A) may perhaps reflect lower levels of AI synthesis and/or secretion in artificial sea water, conditions under which AI production has not been quantified. Finally, by providing exogenous, purified CAI-1 and AI-2 to the chitinous biofilm (as described in Materials and methods), the AI-deficient strain was capable of taking up DNA with a transformation efficiency similar to *V. cholerae* strains that produced their own AIs (Fig. 2.3B).

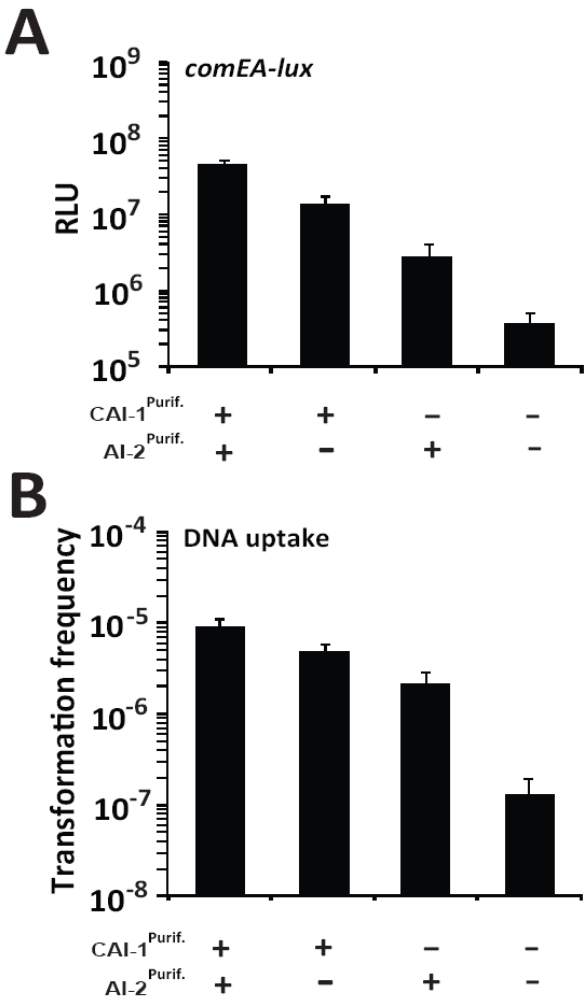

Figure 2.3. Purified AI molecules induce *comEA* expression and DNA uptake in *V. cholerae*. TfoX-induced p*comEA-lux* expression **(A)** and chitin-induced transformation frequencies **(B)** for a *V. cholerae* AI-deficient (Δ*cqsA* Δ*luxS*) strain incubated in the presence of 10 μM purified CAI-1 and 10 μM AI-2 as indicated below the figures and described in Material and methods. Data shown are mean values ± standard deviation from one representative experiment of three performed.

2.4.3. AIs produced by other bacteria in a mixed-species biofilm activate *comEA* expression and DNA uptake by *V. cholerae*

Based on our results with the QS mutants and purified AIs (Figs. 2.2 and 2.3), we hypothesized that *V. cholerae* might also sense and respond to AIs irrespective of their origin, including AIs derived from other *Vibrios* within in a mixed-species biofilm. We reasoned that a mixed-species consortium may more closely reflect conditions in

environmental biofilms that are unlikely to be mono-species in composition (71, 182). To demonstrate the feasibility of a mixed-species, crab-shell microcosm assay, the *V. cholerae* AI-deficient recipient (Δ*cqsA* Δ*luxS*) was co-cultured on chitinous crab shells with *V. cholerae* AI-proficient donor strains that were $HapR^-$ (and thus QS^-) but still capable of producing both AIs, only CAI-1, only AI-2, or neither AI. The AI-deficient *V. cholerae* recipient responded to both AIs derived from *V. cholerae* $HapR^-$ AI-donors within the biofilm and efficiently acquired extracellular DNA. Maximal transformation frequency occurred when the *V. cholerae* AI-recipient was provided with both AIs, while the response to only CAI-1 or only AI-2 was reduced. An AI-donor unable to produce either AI promoted the lowest transformation frequency (Fig. 2.4). Similar results were obtained with several additional *V. cholerae* isolates that served as the CAI-1 and AI-2 donor (data not shown).

These results validated that in the crab-shell microcosm AIs derived from donor *V. cholerae* cells could promote *comEA* expression in a *V. cholerae* recipient, thus we monitored DNA uptake in the *V. cholerae* Δ*cqsA* Δ*luxS* AI-deficient strain, co-cultured in a mixed biofilm with different *Vibrio* species serving as AI donors. Indeed, in these mixed-species biofilms, *V. cholerae* acquired extracellular DNA in response to AIs secreted by bioluminescent *V. harveyi* (Fig. 2.4), which encodes a *V. cholerae* QS pathway (79). As with *V. cholerae*, the maximal transformation frequency occurred with the WT *V. harveyi* strain, which produces both CAI-1 and AI-2. Transformation decreases when only CAI-1, or AI-2 was provided, and was most impaired in the absence of either AI (Fig. 2.4). We also measured transformation frequency of *V. cholerae* AI-deficient recipient in response to WT *V. parahaemolyticus*, and *V. fischeri* AI-donors. Transformation efficiency of these *Vibrio* strains followed a pattern of *comEA-lux* expression that matched the corresponding donor strains; the *V. parahaemolyticus*

strain used produces both CAI-1 and AI-2 and promoted transformation with a frequency similar to *V. harveyi*. The *V. fischeri* strain tested (and another sequenced *V. fischeri* strain, data not shown) only encode for *luxS* (and not *cqsA*), and thus produce AI-2, but not CAI-1. *V. fischeri* poorly promoted DNA uptake by the *V. cholerae* recipient (Fig. 2.4), consistent with AI-2 playing a minor role in natural transformation. Taken together, these observations support a model that *V. cholerae* can switch to the competent state and acquire DNA horizontally in a chitinous environmental biofilm by responding to AI signals derived from members of the multi-species consortium.

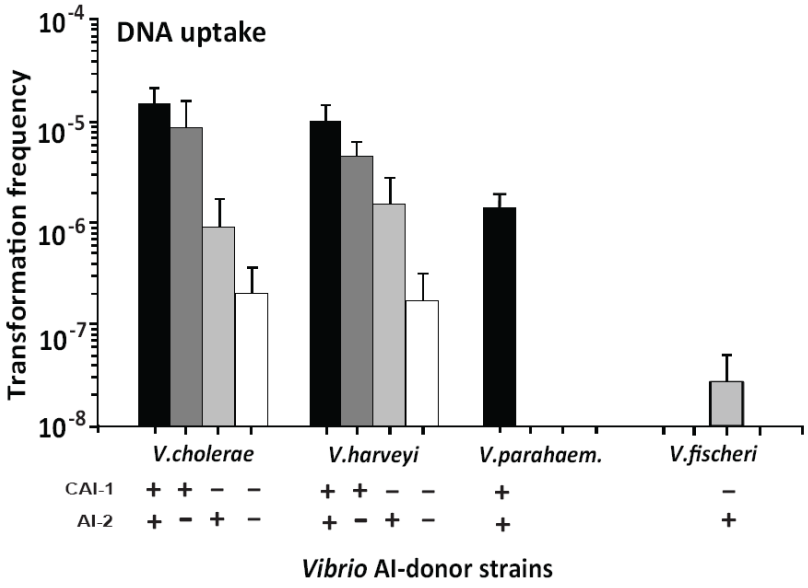

Figure 2.4. AIs derived from different *Vibrio* species induce DNA uptake in *V. cholerae* within a mixed-species biofilm. Chitin-induced transformation frequency of a *V. cholerae* AI-deficient (Δ*cqsA* Δ*luxS*) strain co-cultured with HapR$^-$ (QS$^-$) *V. cholerae* donors and with other *Vibrio* species shown, in triplicate wells carrying crab shell fragments and extracellular DNA. Indicated below figure are the AIs produced by each *Vibrio* species tested. See Material and methods for details. Data shown are the mean transformation frequencies ± the standard deviation of triplicate wells determined from one representative experiment of three performed.

2.5. Discussion

Induction of the competence program in *V. cholerae* requires the chitin-responsive TfoX pathway and the AI-responsive QS pathway. When both systems are functional, DNA uptake machinery facilitates the transport of extracellular DNA into the bacterial cell, where it may be incorporated into the genome by homologous recombination (75). Many *Vibrios* encode for chitin utilization and competence genes (70, 92, 131, 140), which suggests the possibility that natural transformation may be a conserved mechanism for both pathogenic and non-pathogenic *Vibrios* to horizontally acquire virulence and other genes within a community. Recognizing that many *Vibrios* possess a *V. cholerae*-like QS circuits and produce CAI-1 and AI-2, we examined the relationship between AIs production and DNA uptake. Specifically, we showed that (i) *V. cholerae* efficiently activated a *comEA-lux* reporter in response to self-produced AIs as well as purified AIs, and (ii) a *V. cholerae* AI-deficient strain readily acquires DNA when co-cultured with purified AIs and also with AIs produced by other *Vibrio*s within a chitinous mixed-species biofilm. These results support a model that *V. cholerae* can switch to the competent state in a chitinous environmental biofilm by responding to AI molecules derived from members of the multi-species consortium.

Communication via *Vibrio* AI molecules has been studied in many laboratory systems that relied exclusively on cell-free culture fluids or monocultures (18, 89, 120), single-species co-cultures (78), or co-cultures of *Vibrios* with other bacteria unlikely to occupy the same environmental niches (186). These studies were not designed to reflect natural environmental setting that *Vibrios* typically encounter, such as the chitinous surfaces of animals (111). So too, mutants of *V. cholerae (*Δ*hapR* and Δ*luxO)*, which regulate QS-controlled genes irrespective of AI-accumulation, provided the first demonstration of the

role of QS in an animal model of cholera (195), but do not directly demonstrate the role of extracellular AI molecules. Only recently has secreted CAI-1 been shown to repress virulence *in vivo* (56). In a similar manner, we show here for the first time that extracellular CAI-1 and AI-2 molecules directly activate DNA uptake within a mixed-species environmental biofilm. *Vibrio*-specific CAI-1 appears to play a major role, and inter-species AI-2 a minor role, suggesting that induction of DNA uptake may not be restricted exclusively to a response to AIs produced by *Vibrio* species, but that HGT may also be promoted by AI-2 derived from non-Vibrio members of a biofilm. Addition studies will be necessary to determine whether the behavior described here is cooperative "cross-talk" between bacteria, or whether *V. cholerae* simply uses the AI molecules derived from others as a cue to alter gene expression (54). It will also be interesting to determine whether additional chitinous materials that support growth of Vibrios and other bacteria in marine environments (14, 52, 95, 100, 114, 161) also stimulate AI-induced DNA uptake (14).

Recent genomic comparison studies of multiple *V. cholerae* isolates suggest that substantial horizontal gene transfer (HGT) events among *Vibrio* species may account for the presence of large "genomic islands" of transferred DNA (43). Transduction of the cholera toxin genes encoded within a filamentous phage (CTXΦ) permits exchange of virulence factors among *V. cholerae* (177). In laboratory microcosms, DNA encoding antigenic determinants and also carrying CTXΦ occurs via chitin-induced HGT (23, 173) between *V. cholerae*. It is proposed that HGT among *Vibrio* species likely explains the current genome structures, but it has yet to be demonstrated whether chitin-induced HGT can promote DNA exchange among different *Vibrios* in environmental microcosms.

We are currently performing experiments to test a model that AIs may promote inter-species HGT and emergence of genetic diversity in Vibrios.

The major restraint to genetic exchange between species occurs at the level of homologous recombination between the donor extracellular DNA and recipient genomic DNA (86). Recombination between partially homologous DNA depends on the extent and degree of DNA homology, which is monitored by the mismatch repair system (MMR) (153). Genomic comparisons indicate that naturally occurring MMR-deficient environmental "mutator" strains of *V. parahaemolyticus* have increased genetic and phenotypic diversity relative to clinical isolates, suggesting that such mutator strains are also "promiscuous" for inter-species DNA uptake (84). Inactivation of the MMR gene, *mutS,* enhances HGT between *E. coli* and *Salmonella typhimurium* by up to three orders of magnitude (145); likewise a *V. cholerae* Δ*mutS* strain we constructed was indeed capable of inter-species DNA uptake (data not shown). We are currently characterizing collections of environmental *V. cholerae* isolates for MMR, QS, and transformation proficiency to determine the role of AI molecules in the emergence of genetic diversity of these marine bacteria.

2.6. Acknowledgements

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CHAPTER 3

NATURAL COMPETENCE IN V*IBRIO CHOLERAE* IS CONTROLLED BY A NUCLEOSIDE SCAVENGING RESPONSE THAT REQUIRES CYTR-DEPENDENT ANTI-ACTIVATION

Reproduced in part with permission from Antonova E.S., Bernardy E.E., Hammer B.K. Natural competence in *Vibrio cholerae* is controlled by a nucleoside scavenging response that requires CytR-dependent anti-activation. *Mol Microbiol.* 86 (5):1215-31.

3.1. Abstract

Competence for genetic transformation in *Vibrio cholerae* is triggered by chitin-induced transcription factor TfoX and quorum sensing (QS) regulator HapR. Transformation requires expression of ComEA, described as a DNA receptor in other competent bacteria. A screen for mutants that poorly expressed a *comEA*-luciferase fusion identified *cytR*, encoding the nucleoside scavenging cytidine repressor, previously shown in *V. cholerae* to be a biofilm repressor and positively regulated by TfoX, but not linked to transformation. A Δ*cytR* mutant was non-transformable and defective in expression of *comEA* and additional TfoX-induced genes, including *pilA* (transformation pseudopilus) and *chiA-1* (chitinase). In *Escherichia coli*, "anti-activation" of nucleoside metabolism genes, via protein-protein interactions between critical residues in CytR and CRP (cAMP receptor protein), is disrupted by exogenous cytidine. Amino acid substitutions of the corresponding *V. cholerae* CytR residues impaired expression of *comEA, pilA,* and *chiA-1,* and halted DNA uptake; while exogenous cytidine hampered *comEA* expression levels and prevented transformation. Our results support a speculative model that when *V. cholerae* reaches high density on chitin, CytR-CRP interactions "anti-activate" multiple genes, including a possible factor that negatively controls DNA uptake. Thus, a nucleoside scavenging mechanism couples nutrient stress

and cell-cell signaling to natural transformation in *V. cholerae* as described in other bacterial pathogens.

3.2. Introduction

Vibrio cholerae is the bacterium responsible for the fatal diarrheal disease cholera, but it is also a natural inhabitant of marine and estuarine environments where it commonly forms biofilms on abiotic and biotic surfaces, such as chitinous chironomids (non-biting flies) and zooplankton molts (72, 169). Horizontal gene transfer (HGT) of traits among *Vibrios* is thought to promote rapid genetic exchange that is responsible for the mosaic genome structure of *Vibrios* revealed by recent genomic sequencing efforts (43). Competence for genetic transformation in *V. cholerae* was recently reported and represents a newly appreciated mode of HGT for this aquatic bacterium (118). *V. cholerae* natural transformation is induced by two environmental signaling pathways: a quorum sensing system and a chitin utilization system. The network connecting these two systems to natural competence remains poorly understood.

Quorum sensing (QS) enables bacterial populations to collectively control gene expression and thus coordinate behaviors presumably unproductive for individuals (131). Like many other *Vibrio* species, *V. cholerae* populations accomplish QS by producing and responding to autoinducer (AI) signal molecules, specifically two AIs, CAI-I and AI-2 (for review see (79)). At low cell density (LCD) the unbound receptors of CAI-1 and AI-2 (CqsS and LuxP/Q, respectively) behave as kinases and phosphorylate response regulator LuxO, via LuxU. Phosphorylated LuxO activates transcription of four small RNAs, termed Qrr1-4 (Quorum Regulatory RNAs). In association with the RNA chaperone Hfq, the Qrrs base-pair with the mRNAs of several target genes including *hapR*, which encodes the master regulator of QS, HapR (11, 13, 78, 107, 150, 167). At

high cell density (HCD), binding of AIs to their cognate receptors switches them to phosphatases, reversing the phosphorylation cascade and inactivating LuxO. Thus, HapR is produced and activates expression of numerous genes at HCD including *hapA*, which encodes a protease that plays a role in interactions of *V. cholerae* with aquatic hosts (73), and the *comEA* gene, which encodes a periplasmic DNA binding protein shown to be the DNA receptor for transformation in *Bacillus subtilis* (118, 142) (Fig. 3.1). Therefore, wild-type (WT) *V. cholerae* is naturally competent at HCD, a Δ*hapR* mutant is non-transformable, and a Δ*luxO* mutant that constitutively produces HapR takes up DNA independently of cell density (23, 118). So too, a *V. cholerae* strain unable to produce either AI only expresses *comEA* and takes up DNA when provided exogenous AIs (5, 165).

Activation of *comEA* expression by QS AIs requires an additional extracellular signal, namely chitin, the most abundant carbon source in the ocean. Genetic studies (108, 119, 187, 188) support that when chitin is present, $(GlcNAc)_2$ binds to the CBP (chitin binding protein) activating the ChiS sensor kinase, which in turn leads to the production of the Hfq-dependent TfoR sRNA. TfoR promotes translation of the mRNA encoding an ortholog of *Haemophilus influenzae* regulator, Sxy (called TfoX in *Vibrio* species), which appears to promote transcription of competence genes in other bacteria by direct interactions with the cAMP receptor protein, CRP (119, 147) (Fig. 3.1). In *V. cholerae*, experimental induction of *tfoX,* such as from the *tac* promoter, is sufficient to promote transformation and activate expression of the *comEA* and multiple chitinase genes (including *chiA-1*) independent of chitin (5, 118, 188). In addition to *comEA* and *chiA-1*, competence pseudopilus genes (*pilA, pilB, pilQ*) are also under TfoX control (118) (Fig. 3.1). Thus, for *V. cholerae* both the chitin responsive pathway (i.e. TfoX) and QS (i.e. HapR) are required for sufficient *comEA* expression to promote uptake of DNA, as Δ*tfox*

and Δ*comEA* mutants, like a Δ*hapR* mutant, are severely impaired for transformation (118).

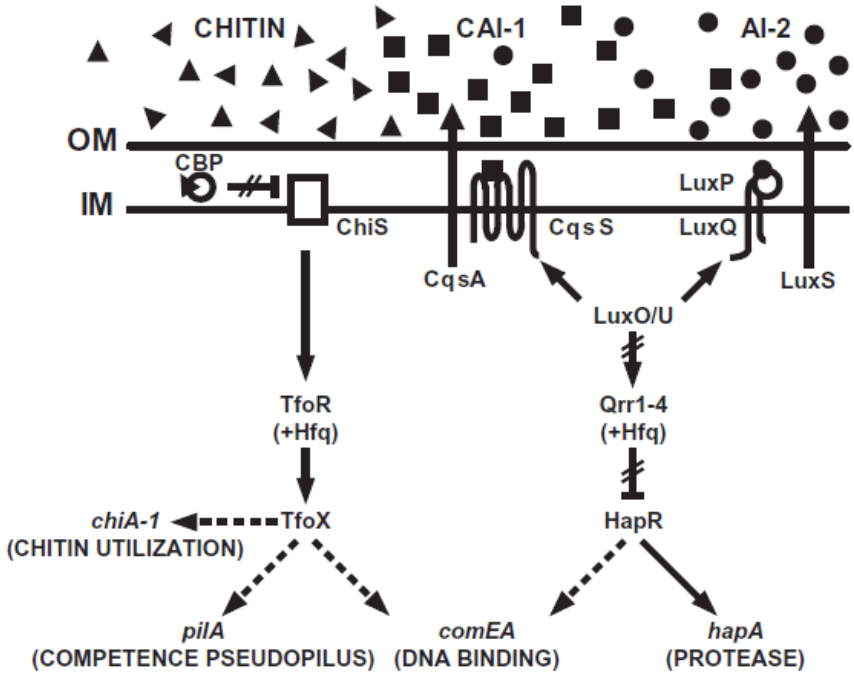

Figure 3.1. Current model for activation of TfoX- and HapR-controlled genes in response to chitin and quorum sensing signal molecules in *V. cholerae*. Chitin binding permits ChiS-dependent transcription of the TfoR sRNA that promotes TfoX translation. Quorum sensing AI accumulation at high cell density triggers dephosphorylation of LuxO (via LuxU), which prevents Qrr1-4 sRNA repression of *hapR* translation. TfoX regulates genes for chitin utilization (*chiA-1*), a competence pseudopilus (*pilA*), and DNA binding and uptake (*comEA*); while HapR positively regulates transcription of *hapA* (protease) and *comEA*. Refer to the text and (118, 131) for details of signal transduction pathways depicted. Dashed lines represent predicted network connections studies here.

DNA uptake by *V. cholerae* was demonstrated in 2005, and more recently in other marine *Vibrios*, such as *V. parahemolyticus*, *V. fischeri*, and *V. vulnificus* (38, 139, 164). Recent genome sequencing efforts, however, predict that not only the Vibrionaceae, but also the Enterobacteriaceae, encode orthologs of many DNA uptake genes, including *tfoX* and *comEA* (30). Despite the fact that many members of the Enterobacteriaceae are not naturally competent, it has been proposed that a common regulatory mechanism for natural competence involving both TfoX and CRP may be shared among these two

families of the γ-proteobacteria (30, 147). Studies that bolster this hypothesis include demonstration that expression of natural transformation genes in *Escherichia coli* and *V. cholerae* is subjected to carbon catabolite repression (CCR) (21, 30, 118, 193). CRP, the global regulator of CCR, in gram negative bacteria, together with its allosteric effector cAMP, controls the expression of multiple genes involved in the utilization pathways of alternative carbon sources when glucose levels in the cell are low (27, 53). In gram positive bacteria, CCR also coordinates a response to low glucose levels but instead utilizes the CcpA transcription factor (178). Interestingly, a recent study demonstrating that CcpA in *Streptococcus* may induce natural competence as a consequence of CCR supports perhaps an even broader role for nutrient starvation in inducing DNA uptake (193).

The complex CRP-mediated response to nutrient stress in gram negative bacteria may also include participation by additional regulatory factors that enable the bacteria to utilize not only varied carbohydrates but also nucleic acids as well. In *E. coli*, for example, many proteins for scavenging of extracellular free nucleosides are encoded by genes in a regulon that is negatively controlled by the CytR regulator (175). The presence of the nucleoside cytidine in the growth medium alleviates repression by CytR, which works in conjunction with CRP in *E. coli* as an “anti-activator” of a subset of CRP-activated genes that are involved in nucleoside transport and metabolism. In *V. cholerae,* CytR has been shown to repress expression of the *udp* gene for nucleoside catabolism in nucleoside-poor environments, in addition to impairing biofilm development by unknown mechanisms (83). Moreover, several studies in *V. cholerae* demonstrated that CRP is involved in QS, biofilm formation, motility, and cholera toxin production, as well as natural competence (21, 29, 61, 109, 110). While CRP may well participate in many steps of a regulatory network for DNA uptake, a molecular mechanism linking

CRP to the control of natural competence genes has not been demonstrated in *V. cholerae*. Here we provide evidence that the CytR anti-activator, which requires CRP to function, coordinates a nutrient scavenging response in *V. cholerae* that controls natural competence.

3.3. Experimental procedures

3.3.1. Bacterial strains, plasmids, and culture conditions

The relevant genotypes of the *V. cholerae* strains and plasmids used in the study are listed in Table 3.1. *V. cholerae* strains were incubated at 37°C on Luria-Bertani (LB) agar, and in LB broth with shaking. AB minimal medium (44) modified to include 0.7 mM Na_2SO_4 (Waters, C.M., unpublished) was used for bioluminescence assays where noted. Artificial sea water (ASW; Instant Ocean) was used for chitin-induced natural transformation assays as described previously (5). Antibiotics (Fisher BioReagents) chloramphenicol (Cm), kanamycin (Kan), and streptomycin (Str) were used at concentrations of 10, 100, 5000 μg ml^{-1}, respectively. Expression of the *cytR* gene encoded on p-*tac*-*cytR* was induced with 0.5mM isopropyl-b-D-thiogalactopyranoside (IPTG; Fisher BioReagents). Where noted, *V. cholerae* cultures were supplemented with 100mM of cytidine or deoxycytidine (Sigma).

Table 3.1. *V. cholerae* strains and plasmids used in this study

Strains	Genotype or description	Reference
V. cholerae strains		
C6706str	El Tor biotype, O1; $HapR^+$	(170)
EA305	*tfoX** (*tfoX* controlled by *tac* promoter)	This study
SLS349	Δ*luxO*	(179)
EA281	Δ*luxO, tfoX**	This study
EA349	Δ*luxO,* Δ*lacZ*::*hapR, tfoX**	This study
EA407	Δ*luxO,* Δ*lacZ*::*hapR, tfoX*, cytR*::Tn*5*	This study
BH1543	Δ*hapR*	(5)
EA307	Δ*hapR, tfoX**	This study
EA408	Δ*cytR*	This study
EA410	Δ*cytR, tfoX**	This study
EA415	Δ*luxO,* Δ*cytR*	This study
EA636	Δ*luxO,* Δ*cytR, tfoX**	This study
EA517	Δ*cytR,* Δ*lacZ*::c*ytR*, *tfoX**	This study
EA605	*cytR-L161A*	This study
EA606	*cytR-L161A, tfoX**	This study
EA680	*cytR-D273N*	This study
EA682	*cytR-D273N, tfoX**	This study
EA577	Δ*crp*::Kan^R	This study
EA601	Δ*crp*::Kan^R, *tfoX**	This study
MN171	Δ*crp*::Kan^R, Δ*cytR*	This study
MN173	Δ*crp*::Kan^R, Δ*cytR, tfoX**	This study
EA090	Δ*lacZ*::Kan^R	(5)
Plasmids	**Features**	**Reference**
pBBRlux	Cloning vector, Cm^R	(107)
pEA209	*comEA-lux*, pBBRlux-based, Cm^R	(5)
pEA493	*pilA-lux*, pBBRlux-based, Cm^R	This study
pEA495	*chiA-1-lux*, pBBRlux-based, Cm^R	This study
pEA603	*udp-lux*, pBBRlux-based, Cm^R	This study
pBBRlux-hap	*hapA-lux*, pBBRlux-based, Cm^R	(13)
pEA500	p-*tac-cytR*, pEVS143-based, Kan^R	This study

3.3.2. DNA manipulations

Standard protocols were used for all DNA manipulations (151). Restriction enzymes, T4 DNA ligase (New England Biolabs), and Phusion DNA polymerase (Finnzymes) were used for cloning and PCR reactions. In frame deletions, amino acid substitutions, and insertion mutants in *V. cholerae* were constructed by allelic exchange pKAS32-based plasmids (159). Genomic DNA marked with Kan^R gene at the *lacZ* locus (5) was extracted using a ZR Fungal/Bacterial DNA kit^{TM} (Zymo Research) for chitin-induced natural transformation assays. Plasmids carrying the luciferase-based transcriptional reporters (*comEA-lux*, *pilA-lux*, *chiA-1-lux*, and *udp-lux*) were constructed as previously described (5). Briefly, the promoter and a portion of the coding region of corresponding gene from WT *V. cholerae* was PCR amplified with an upstream primer containing a SpeI site and a downstream primer containing a BamHI site, and then cloned into the SpeI/BamHI-digested pBBRlux vector (107). The IPTG-inducible p-*tac-cytR* plasmid was constructed by amplifying the entire coding region of *vc2677* and cloning it into the pEVS143 vector by insertion into the EcoRI and BamHI restriction sites.

3.3.3. Transposon mutagenesis of *V. cholerae*

The suicide delivery plasmid pRL27 encoding the Tn*5* transposon conferring resistance to kanamycin (Kan^R) (Larsen et al, 2002) was transferred by conjugation from *E. coli* S17λpir to the *V. cholerae ΔluxO, tfoX** recipient strain merodiploid for *hapR* (Table 3.1, EA349). Three independent pools of ~10^5 Tn*5* Kan^R *V. cholerae* mutants were conjugated with an *E. coli* S17λpir donor carrying the *comEA-lux* reporter plasmid to create a library of transposon mutants. Kan^R transconjugant mutant colonies were arrayed to microtiter plates with a Genetix QPix2^{XT} colony picker followed by screening

for candidates with defective *comEA-lux* expression using a BioTek multimode plate reader. The identity of candidate target genes found in the screen was determined by BLAST analysis to the *V. cholerae* C6706 genome of the DNA sequences adjacent to the Tn*5* insertion, by standard methods described previously (80, 106).

3.3.4. Bioluminescence assay

V. cholerae bioluminescence expression was assayed as described previously (Miller et al, 2002, Zhu et al, 2002). *V. cholerae* strains carrying a lux-based reporter plasmid were incubated in LB at 37°C overnight with appropriate antibiotics and IPTG where noted, and bioluminescence and absorbance were quantified thereafter. For bioluminescence measurements in the presence of cytidine, *V. cholerae* strains carrying a reporter plasmid were incubated overnight for 9-11 h at 37°C in AB minimal media containing appropriate antibiotics and supplemented with 100 mM cytidine. A chitin tile was added to each tube to provide a carbon source and for induction of *tfoX*. Bioluminescence was measured using a Wallac model 1409 liquid scintillation counter as described previously (78). The optical density of each culture was measured with a spectrophotometer. Relative Light Units (RLU) are defined as counts per min^{-1} ml^{-1}/OD_{600}. Single-time-point experiments were performed with triplicate samples.

3.3.5. Chitin-induced natural transformation assay

Chitin-induced transformation experiments were performed as described previously (118). 2µg of *V. cholerae* genomic DNA marked with a Kan^R gene at the *lacZ* locus was used as an extracellular DNA source. For enumeration of transformants, cultures were plated onto LB medium containing Kan. Transformation frequency was defined as Kan^R CFU ml^{-1}/total CFU ml^{-1}. To measure transformation frequencies of *V. cholerae* strains in

the presence of cytidine, ASW medium was supplemented with 100mM cytidine or 100 mM deoxycytidine. Independent experiments were performed in triplicate.

3.4. Results

3.4.1. Identification of a competence-deficient *V. cholerae* mutant

Transcription of *comEA* depends on induction of TfoX in response to chitin, and on HapR, which is produced as a result of accumulated quorum sensing (QS) autoinducers (AIs) at high cell density (HCD). *V. cholerae* strains that do not produce TfoX, or are unable to synthesize QS AIs, and thus produce no HapR, are severely impaired for *comEA* expression (5, 118, 119, 165). To identify one or more positive regulators of the competence gene, *comEA,* in *V. cholerae,* we performed a transposon mutagenesis of El Tor *V. cholerae* strain C6706 using a well-described Tn*5* system (106). We eliminated the requirement of chitin by first constructing a derivative of C6706 that expresses the *tfoX* gene from the chromosome under control of a constitutive *tac* promoter (referred to here as a *tfoX** allele). C6706 has a frame shift mutation in *lacI* and does not encode a functional LacI protein, thus *tfoX* is not LacI-repressed in this strain. Maximal expression of a *comEA-lux* reporter that we constructed previously (5) was observed in the *tfoX** strain at HCD, when the QS response results in HapR production (Fig. 3.2A, bar 1). A deletion of *hapR* in this background results in a reduction in *comEA* expression by ~10,000 fold, as expected (Fig. 3.2A, bar 2). In contrast, a *ΔluxO*, *tfoX** strain expresses *comEA* to levels similar to the *tfoX** strain at HCD, but does so independent of chitin and AI accumulation (data not shown). We further introduced a second copy of *hapR* at the *lacZ* locus into the *ΔluxO*, *tfoX** strain to minimize screening of mutants with Tn*5* insertion in *hapR*, or spontaneous *hapR* mutations that can occur in a *ΔluxO* strain (77). As expected, the merodiploid behaved in a manner indistinguishable from the isogenic

ΔluxO, *tfoX** strain, expressed *comEA* maximally (Fig. 3.2A, compare bar 1 and 3), and was used as the recipient for Tn*5* transposon mutagenesis.

Three independent pools of ~20,000 Tn5 kanamycin^R (Kan^R) mutants were screened for defective *comEA-lux* expression, and one isolate was identified that expressed *comEA* as poorly as a *ΔhapR* mutant (Fig. 3.2A, bar 4). The location of the Tn*5* insertion mapped to *vc2677*, which is annotated in the database as a transcriptional repressor of the LacI family. In 2002, v*c2677* was shown to encode CytR, which represses biofilm formation in *V. cholerae* strain MO10 (83). Importantly, it was later identified by Meibom *et al.* in a microarray study as one of ~100 TfoX-induced genes (118). Because it was predicted to participate in nucleoside metabolism and DNA uptake based on genomic comparison to other naturally competent bacteria (30), we sought to determine a role for CytR in natural competence of *V. cholerae.*

3.4.2. CytR positively regulates *comEA* expression and DNA uptake in *V. cholerae*

To confirm that the defect in *comEA* expression in the transposon-insertion mutant was indeed the result of *cytR* inactivation, we introduced an in-frame *cytR* gene deletion (Δ*cytR*) into various genetic backgrounds of *V. cholerae* using standard methods (159), and measured expression of *comEA-lux* (Fig. 3.2A) and transformation frequency (Fig. 3.2B) of each strain. A Δ*cytR, tfoX** mutant was 10,000 fold reduced for *comEA* expression compared to the WT strain carrying *tfoX** (Fig. 3.2A, compare bar 5 to bar 1), and similar to the *comEA* defect of the Δ*hapR, tfoX** mutant (Fig. 3.2A, compare bar 5 to bar 2). Similarly, a Δ*luxO, tfoX*,* Δ*cytR* mutant was also deficient in *comEA* expression compared to the isogenic Δ*luxO, tfoX** mutant and to the Δ*luxO, lacZ::hapR, tfoX** mutant (data not shown, and Fig. 3.2A, compare bar 6 to bar 3). The Δ*cytR, tfoX** strain

was fully complemented either by insertion a copy of the *cytR* gene under control of its native promoter into the chromosome at the *lacZ* site (Fig. 3.2A, compare bar 7 to bar 1), or by introducing the same *cytR* gene on a plasmid (data not shown), as *comEA-lux* expression was restored to levels observed with the *tfoX** strain.

Transformation frequencies of corresponding *V. cholerae* strains that do not carry a *tfoX** allele, but rather require chitin for *tfoX* expression, were measured by a crab-shell microcosm system described previously (118). The results were consistent with *comEA-lux* expression (Fig. 2A, B). Specifically, no transformants were detected for the Tn*5* mutant, Δ*cytR* mutant, and the Δ*luxO,* Δ*cytR* double mutant, similar to the Δ*hapR* strain. The DNA uptake level of the complemented strain was similar to WT, the Δ*luxO* mutant, and the Δ*luxO, lacZ::hapR* mutant of *V. cholerae* (Fig. 2B, and (5)). These results confirm the role of CytR as a positive regulator of natural competence of *V. cholerae*.

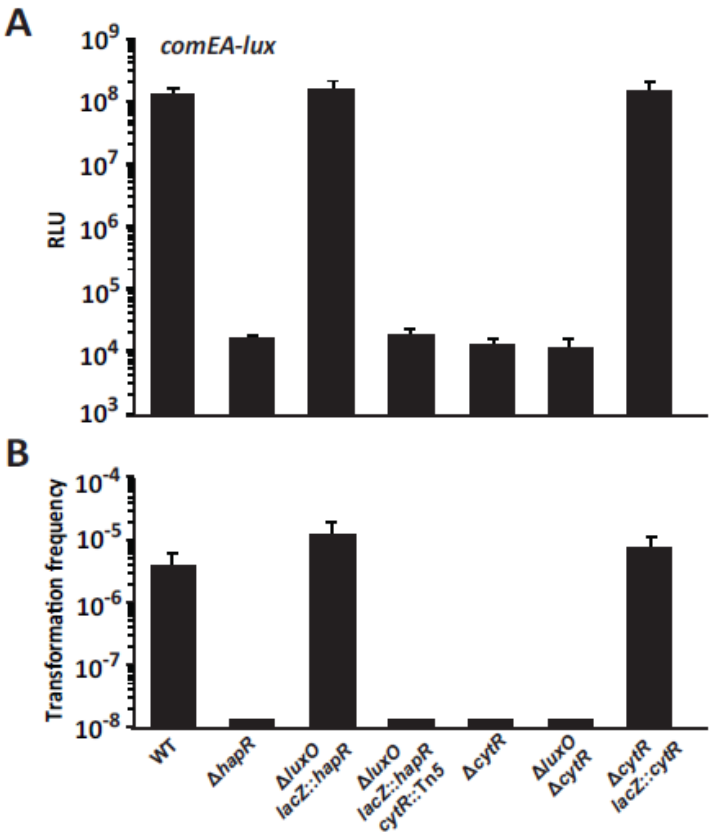

Figure 3.2. CytR regulates *comEA-lux* expression and DNA uptake in *V. cholerae.* **(A)** Triplicate cultures of various *V. cholerae* strains expressing chromosomal *tfoX* gene under control of a constitutive *tac* promoter (*tfoX**) and carrying a plasmid-borne *comEA-lux* reporter were incubated overnight and analyzed for luciferase production. Bioluminescence is defined as relative light production per OD_{600} (RLU). **(B)** Chitin-induced transformation frequency was calculated (see Material and methods) for each *V. cholerae* strain (carrying the native *tfoX* allele) incubated with extracellular DNA in triplicate wells containing crab shell tabs. The limit of detection is 1.0×10^{-9}. Data shown are mean values ± standard deviation from one representative experiment of three performed.

3.4.3. CytR and QS regulate expression of multiple TfoX-induced genes

Based on prior microarray studies, in addition to the *cytR* gene itself, three groups of genes have been shown to be under TfoX control in *V. cholerae*: DNA binding and processing genes (such as *comEA*), chitin degradation and utilization genes (such as *chiA*-1), and competence pseudopilin genes (such as *pilA*) (118). To test whether genes in each of these categories are also regulated by CytR and/or QS, we constructed

luciferase-based transcriptional fusions of representative genes in each group and quantified expression in Luria broth (LB medium) without chitin for *V. cholerae* WT (QS^+, $CytR^+$), Δ*hapR* (QS^-, $CytR^+$), and Δ*cytR* (QS^+, $CytR^-$) strains that carry the constitutive t*foX** allele ($TfoX^+$) or the native *tfoX* allele ($TfoX^-$) that requires chitin for induction. Consistent with microarray studies, expression of all three genes, *comEA*, *chiA*-1, and *pilA*, increased in WT *V. cholerae* in the presence of the *tfoX** allele (~115-, 55-, 15-fold, respectively), which confirms that they are activated by TfoX (Fig. 3.3A, B, C, compare first two bars). However, expression of all three genes in a Δ*hapR* and a Δ*cytR* mutant was approximately as low as in the strains lacking constitutive TfoX, which suggested that not only *comEA*, but also *chiA*-1, and *pilA* are positively regulated by both HapR and CytR (Fig. 3.3A, B, C).

As a control, we also constructed a *lux*-based transcriptional fusion to the *V. cholerae* *udp* gene, encoding uridine phosphorylase, which is repressed by CytR (83). As expected, the transcription pattern of *udp-lux* was reciprocal of the *comEA-lux* results (Fig. 3.3D). Namely, expression decreased in the WT strain when *tfoX* was expressed (Fig. 3.3D, compare the first two bars). In a Δ*hapR* mutant, a similar pattern was observed with slightly greater *udp* expression than the WT strain in the absence of TfoX. Levels of *udp* in the Δ*hapR* strain without *tfoX** were slightly higher than in WT (Fig. 3.3D, compare the 2^{nd} white bar to the 1^{st} white bar), as described prior (109, 190). These results suggest that HapR not only positively regulates *comEA, pilA,* and *chiA-1*, but also negatively regulates *udp* in a manner independent of TfoX induction (and CytR) (Fig. 3.1). Moreover, in a Δ*cytR* mutant, repression of *udp-lux* was eliminated (Fig. 3.3D), consistent with studies that showed repression of the *udp* gene by CytR in *V. cholerae* (83, 196).

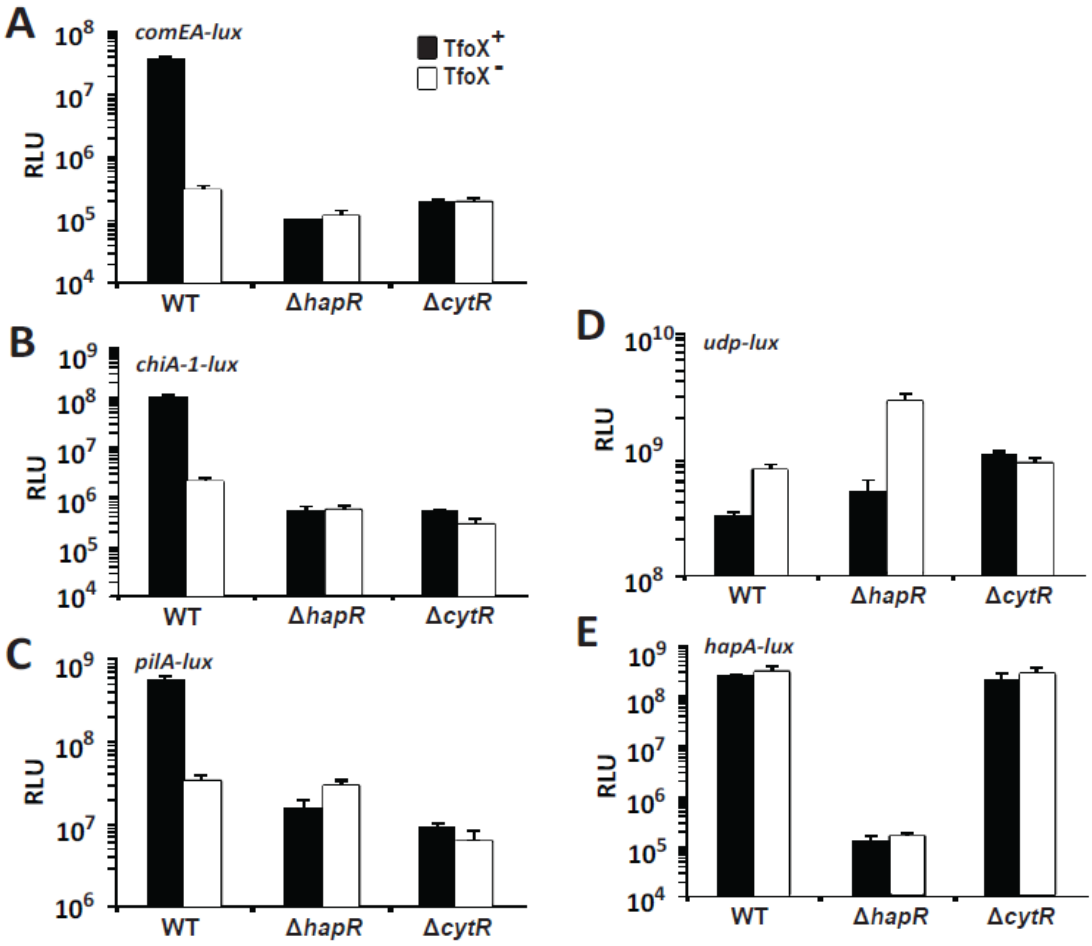

Figure 3.3. CytR positively regulates multiple genes for numerous DNA uptake and chitin utilization. *V. cholerae* strains carrying chromosomally-encoded *tfoX* under control of a constitutive *tac* promoter (TfoX$^+$, black bar) or under its native promoter (TfoX$^-$, white bar) were analyzed for expression of bioluminescence from plasmid-borne *comEA-lux* **(A)**, *chiA-1-lux* **(B)**, *pilA-lux* **(C)**, *udp-lux* **(D)**, and *hapA-lux* **(E)** transcriptional fusions. Bioluminescence is defined as relative light production per OD_{600} (RLU). Data shown are mean values ± standard deviation for the triplicate cultures from one representative experiment of three performed.

As an additional control, we measured the expression pattern of the *hapA* gene, which encodes a haemagglutinin protease that is positively regulated by HapR (13, 77), but not predicted to be under control of either TfoX or CytR. The HapR-dependent expression pattern of *hapA-lux* was as described prior (77). The maximal transcription of *hapA* was observed in both WT and Δ*cytR* mutants, and minimal in a Δ*hapR* mutant and, as predicted, independent of TfoX and CytR (Fig. 3.3E). These results confirm that

regulation of *hapA* is QS-dependent and CytR-independent. Taken together, these data indicate that CytR positively regulates genes controlling the natural competence and chitin utilization network in *V. cholerae,* and as it was shown previously, negatively regulates the *udp* gene involved in nucleoside metabolism.

3.4.4. *V. cholerae* CytR behaves like a CRP-dependent anti-activator

Extensive studies in *E. coli* have demonstrated that CytR associates with global cAMP receptor protein, CRP, to inhibit transcription of a subset of promoters activated by CRP (for review see (175)). For example, the CRP-dependent promoter of *udp* in *E. coli* contains both a distal (CRP-2) and a proximal (CRP-1) binding site allowing activation of *udp* transcription by CRP in the absence of glucose when intracellular cAMP levels are high (26, 196). Specifically, binding of a CRP dimer at the 2-fold symmetric CRP-2 site and at the CRP-1 site, which overlaps the -35 region, positions each CRP dimer to recruit RNA polymerase (RNAP) (Fig 3.4A, top). The CRP consensus site in *E. coli* is depicted in Fig. 3.4B. Optimal spacing of 52-53 bp between the CRP-2 and CRP-1 sites of several CRP-dependent promoters, including *udp*, ensures that a CytR dimer inhibits initiation of transcription of such genes by protein-protein interactions with each CRP dimer. Interaction of CytR with a highly degenerate operator site between the two CRP sites plays a minor role (28), and indeed overexpression of *E. coli* CytR that lacks a DNA-binding domain can still repress the *deoP* promoter, which is also under CytR-CRP control (138, 162). Thus, CytR-CRP interactions are necessary for “anti-activation” of genes in *E. coli*, including *udp*, which are involved in nucleoside scavenging in the absence of preferred carbon sources (Fig. 3.4A, bottom) (175). As a result, we sought to test whether a CRP-dependent CytR anti-activation mechanism may control natural competence in *V. cholerae*, and if such a mechanism was consistent with prior

observations that a Δ*crp* mutant is defective in transformation and that the presence of glucose inhibits chitin-induced DNA uptake (21, 118).

To determine whether the CytR-CRP protein-protein interactions important for the *E. coli* nucleoside scavenging response play a role in *V. cholerae* natural competence, we first examined the degree of similarity between the regulatory proteins involved. It has been shown previously that *V. cholerae cytR* complements an *E. coli cytR* mutant, confirming that it is a functional homolog (83). Protein alignments indicated that *V. cholerae* CytR and CRP are 81% and 98% similar (65% and 95% identical), respectively, to their *E. coli* homologs (Fig. 3.4C). Importantly, alignment revealed that specific residues of *E. coli* CytR, notably residues L169 and F173 (corresponding to L161 and F165 in *V. cholerae*) that form a patch on the surface of CytR crucial for the CytR-CRP interactions in *E. coli* (99), are conserved in *V. cholerae* CytR (Fig. 3.4C). As expected, residues that are components of a corresponding patch on the surface of CRP are also conserved between these nearly identical proteins (not shown). Thus, to determine whether a CytR-CRP anti-activation mechanism is conserved and responsible for natural competence in *V. cholerae,* we measured expression of our *lux*-based gene fusions in a WT *V. cholerae* control strain expressing *tfoX** and compared each to expression levels in an isogenic strain with an in-frame *cytR* gene deletion (Δ*cytR*), or with an L161A amino acid substitution in CytR (*cytR*^{L161A}), which corresponds to the *E. coli* L169A CytR mutation that abolishes CytR-dependent anti-activation (99). Based on the results of the genetic screen with *comEA* (Fig. 3.2A), the expression pattern of QS- and CytR-dependent reporters, *comEA*, *chiA*-1, and *pilA* in the control strains was as expected. Namely, the maximal transcription of each gene was observed in the WT strain carrying the *tfoX** allele, with minimal expression of each gene fusion in an isogenic Δ*cytR, tfoX** strain (Fig. 3.3 and Fig. 3.4D-F; compare bar 1 to bar 2). We reasoned that the *V. cholerae*

*tfoX** strain expressing *cytR*^{L161A} would behave like a *cytR* mutant, because a L161A amino acid substitution in CytR prevents CytR-CRP protein-protein interaction. As predicted, a *tfoX**, *cytR*^{L161A} mutant was severely impaired for expression of *comEA* and *chiA-1*, like the isogenic Δ*cytR* strain (Fig. 3.4D, E). Expression of *pilA* was slightly higher (3-fold) in the *tfoX**, *cytR*^{L161A} double mutant compared to the Δ*cytR* strain, but was still over 10-fold lower than the *tfoX** mutant that expresses *pilA* maximally (Fig. 3.4F; compare bar 2 to bars 3 and 1). We also measured expression levels of *comEA*, *chiA-1* and *pilA* in a *cytR*^{F165A}*, tfoX** strain and observed similar alterations in expression (data not shown), consistent with *E. coli* studies (99).

Previous studies predicted and also documented a role for carbon catabolite repression (CCR) in regulating transformation by *V. cholerae*, and a requirement of CRP for this process (21, 30, 118). We identified CytR as a critical regulator for transformation and expression of multiple competence genes in *V. cholerae* (Figs. 3.2 and 3.3). The impact of the loss of CytR depends on CRP in *E. coli*, thus, to test whether CRP is also required for the observed effects of CytR in *V. cholerae*, we measured *comEA*, *chiA-1* and *pilA* expression in a *V. cholerae tfoX*,* Δ*crp* mutant that constitutively expressed *tfoX*, but carried an in-frame deletion in *crp*. It is important to note that the *V. cholerae* strain used does not encode a functional LacI, and the *tfox** allele is controlled here by the *tac* promoter, which is also insensitive to catabolite repression. The Δ*crp, tfoX** double mutant showed a level of expression intermediate between the WT (*tfoX**) and Δ*cytR, tfoX** strains for all three gene fusions tested (Fig. 3.4D-F, bar 4).

To confirm that CytR function in *V. cholerae* requires CRP, we designed an epistasis experiment test to provide additional evidence that competence gene expression and DNA uptake require CytR-CRP interaction. Specifically, we predicted that the Δ*crp*

mutation, which resulted in intermediate levels of competence gene expression would be epistatic to the Δ*cytR* mutation which produced minimal levels of expression for *comEA*, *pilA* and *chiA-1*. We constructed a Δ*cytR*, Δ*crp*, *tfoX** mutant and measured expression of each of these gene fusions. Indeed, the Δ*cytR*, Δ*crp*, *tfoX** mutant displayed intermediate levels of expression for each of the three gene fusions; like the Δ*crp*, *tfoX** mutant (Fig. 3.4D-F, compare bar 5 to bar 4), and unlike the minimum values observed in the Δ*cytR, tfoX** mutant (Fig. 3.4D-F, compare bars 5 to bar 3).

While the genetic analysis supported a potential model that three TfoX-activated genes previously described (118) are positively controlled by CytR-CRP regulation, this regulation is likely indirect since CytR-CRP anti-activates, or effectively represses, promoters under their direct control (see Discussion). In contrast, the *udp* gene in *V. cholerae* is controlled by CytR*,* like in *E. coli* (83, 196). Indeed, upstream of the *V. cholerae udp* gene is a well conserved distal CRP-2 site centered at -154 separated by 52 bp from a proximal CRP-1 binding site centered at -102, relative to the ATG start codon (Fig. 3.4B) (196). Thus, we predicted that the expression pattern of a *udp-lux* gene fusion would be the reciprocal to that observed for the competence genes in *V. cholerae*; namely, *udp-lux* expression would be higher in a *cytR*^{L161A} mutant, as it is in a Δ*cytR* mutant. As already shown in Fig. 3.3, *udp* expression was lowest in the *tfoX** strain, and maximal (although only ~ 5-fold higher) in a *tfoX**, Δ*cytR* strain. As predicted, the *tfoX**, *cytR*^{L161A} mutant had a *udp* expression level similar to the Δ*cytR* mutant and still higher than the *tfoX** strain (Fig. 3.4G). Surprisingly, the Δ*crp* mutants did not appear to have an intermediate level of expression, perhaps due to the difficulty of resolving difference of less than 3-fold with *lux*-based gene fusions as described prior (76). While the fold differences in *udp-lux* expression observed here in *V. cholerae* were not identical to observations by Zolotukhina *et al.* with a *lacZ* transcriptional fusion of *V.*

cholerae udp measured in *E. coli* (196), the pattern in each case is consistent with negative regulation of *V. cholerae udp* by CytR.

Productive CytR-CRP interactions yield maximal expression of the three competence and chitin utilization genes tested (Fig. 3.4D-F), thus we predicted that DNA uptake would likely follow a similar pattern in transformation assays with corresponding *V. cholerae* strains that expressed *tfoX* in the presence of chitin, rather than in response to a constitutive *tfoX** allele. Indeed, transformation frequencies were consistent to the expression of QS- and CytR-dependent reporters (Fig. 3.4H). Compared to the WT strain that has a transformation frequency of ~10^{-5}, a Δ*cytR* and $cytR^{L169A}$ mutants were non-transformable; as were the Δ*crp* and Δ*cytR*, Δ*crp* mutants. These observations are consistent with previous studies (21, 118), and confirm the essential role of CytR-CRP interactions in natural competence of *V. cholerae.* Specifically, they suggest that productive protein-protein interaction between these two regulators ensures the expression of genes for DNA uptake in nutrient poor settings where glucose is absent, but the alterative carbon source, chitin, is abundant.

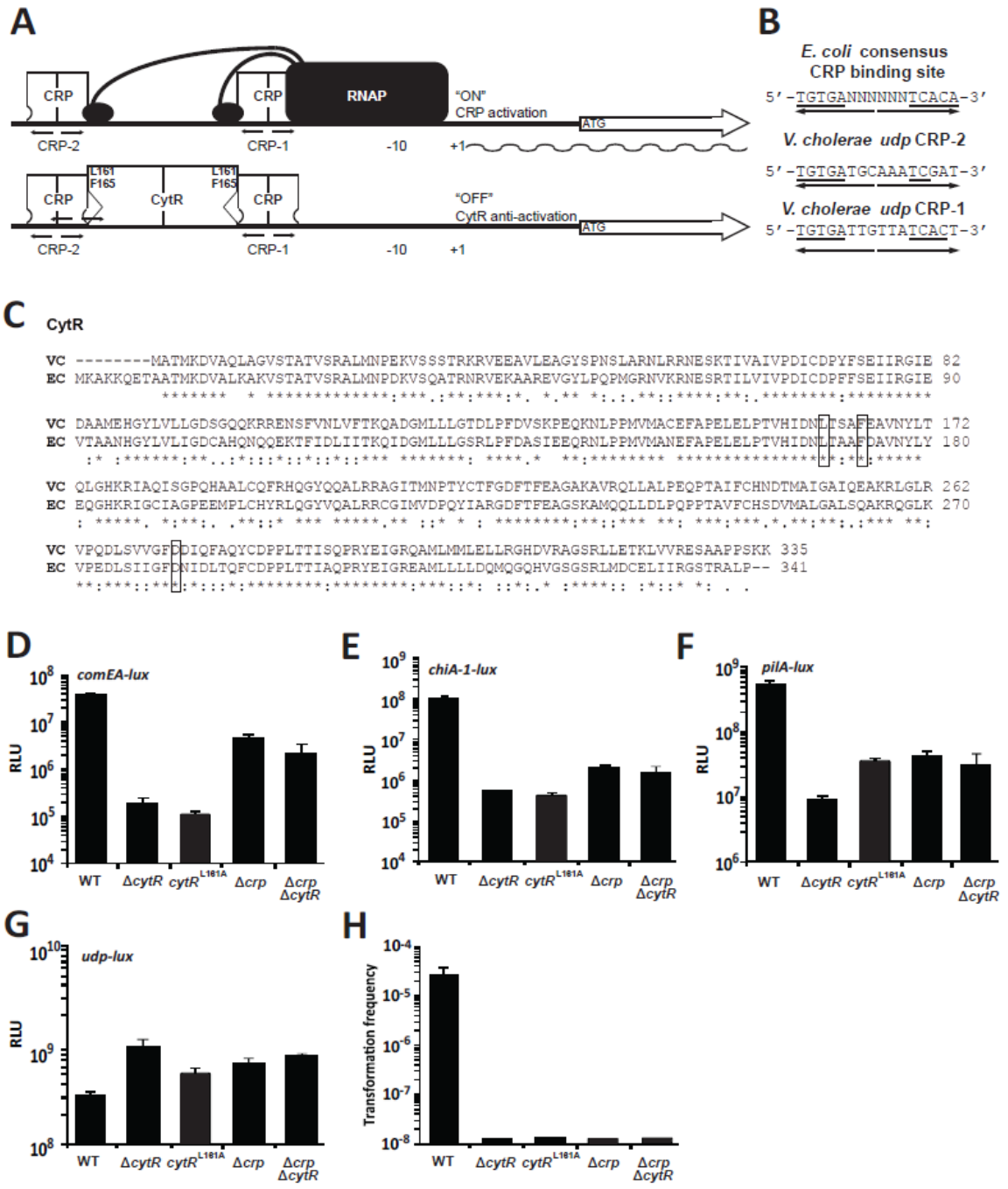

Figure 3.4. *V. cholerae* CytR is a CRP-dependent anti-activator. (A) Top: Model of promoter region of *V. cholerae* genes transcriptionally activated when a CRP dimer binds to both a proximal (CRP-1) and distal (CRP-2) binding site, and recruits RNA polymerase by association with each αCTD of RNAP (black ovals). Bottom: CytR makes protein-protein interactions via L161 and F165 with each CRP dimer blocking CRP-dependent activation of transcription at the promoter via an "anti-activation" mechanism. Arrows at each CRP binding site highlight that the sequence is an inverted repeat (adapted from (28)). **(B)** The *E. coli* consensus binding site for one CRP dimer, and the predicted distal (CRP-2) and proximal (CRP-1) binding sites in the *udp* promoter region

of *V. cholerae*. Arrows highlight the inverted repeat sequence, and underlined nucleotides indicate the critical nucleotides of the 5'-TGTGA(N_6)TCACA-3' consensus. **(C)** Alignment of the amino acid sequences of *V. cholerae* and *E. coli* CytR. Boxed amino acids indicate important resides for CytR-CRP interaction (L161 and F165) and for cytidine induction (D273) as described in text. **(D-G)** Expression of *comEA-lux* (D), *chiA-1-lux* (E), *pilA-lux* (F), and *udp-lux* (G) transcriptional fusions in *V. cholerae* strains carrying chromosomal *tfoX* under control of a constitutive *tac* promoter (*tfoX**). Bioluminescence is defined as relative light production per OD_{600} (RLU). Data shown are mean values ± standard deviation for the triplicate cultures from one representative experiment of three performed. **(H)** Transformation frequency for *V. cholerae* mutants described in D-G. **(I)** Model for the role of putative repressor X in *V. cholerae*. Left: CytR-CRP anti-activation of the X repressor results in minimal expression of *comEA*. Middle: In a Δ*cytR* mutant, CRP activates *comEA* maximally. Right: In a Δ*crp* mutant the lack of CRP activation results in intermediate basal *comEA* expression levels.

3.4.5. CytR overexpression is not sufficient for maximal *comEA* expression

Previously, Meibom *et al.* demonstrated that not only were *comEA*, *pilA* and *chiA-1* positively regulated by TfoX, but also *cytR* as well (118). Because we had identified *cytR* in a screen with a strain that expressed TfoX and HapR constitutively (Fig. 3.2), we sought to determine whether CytR may fit in a regulatory pathway downstream of TfoX, or possibly HapR. To test this we first constructed a *cytR-lux* fusion, however we observed no change in expression when we compared levels in a WT strain to strains carrying a *tfoX** allele, or Δ*tfoX* and Δ*hapR* strains (data not shown). As an additional test to determine whether CytR may be under control of TfoX, we constructed a plasmid (p-*tac-cytR*) to control transcription of the *cytR* gene by the IPTG-inducible, *tac* promoter. We reasoned that if the only role of TfoX, when induced by chitin (mimicked by the *tfoX** allele), was to activate *cytR* transcription, then a strain carrying p-*tac-cytR* (+IPTG) would express *comEA* to maximal levels sufficient for DNA uptake, even in a Δ*tfoX* strain. Alternatively, if CytR acts on competence gene expression in a manner that is independent of TfoX, then *tfoX** would still be required for maximal *comEA* expression in strains over-expressing cytR from the p-*tac-cytR* plasmid. As Figure 3.5 shows, in the absence of chitin, WT *V. cholerae* (with the native *tfoX* allele) carrying the p-*tac-cytR*

plasmid expresses *comEA* minimally in the absence of IPTG, and reach an intermediate level of *comEA* upon IPTG induction of *cytR* (Fig. 3.5, 1st set of bars). In a Δ*cytR* mutant, and a Δ*tfoX* mutant, a similar modest increase in *comEA* expression (10-fold) was also observed upon *cytR* expression by IPTG from p-tac-cytR (Fig. 3.5, 2nd and 3rd set of bars). However, the 100-fold increase in *comEA* expression to the maximal level of >1.0 x 10^7 was only achieved in strains carrying *tfoX** strain (Fig. 3.5, 4th and 5th sets of bars). Consistent with the epistasis test shown in Fig. 3.4, the Δ*cytR*, Δ*crp* and Δ*cytR*, Δ*crp*, *tfoX** strains express intermediate levels of *comEA* that are not altered by CytR overexpression (Fig. 3.5, 6th and 7th sets of bars). These results suggest that *cytR* has a positive impact on competence gene expression in the absence of TfoX, however, an independent contribution by TfoX in required to achieve the levels of competence gene expression necessary for DNA uptake.

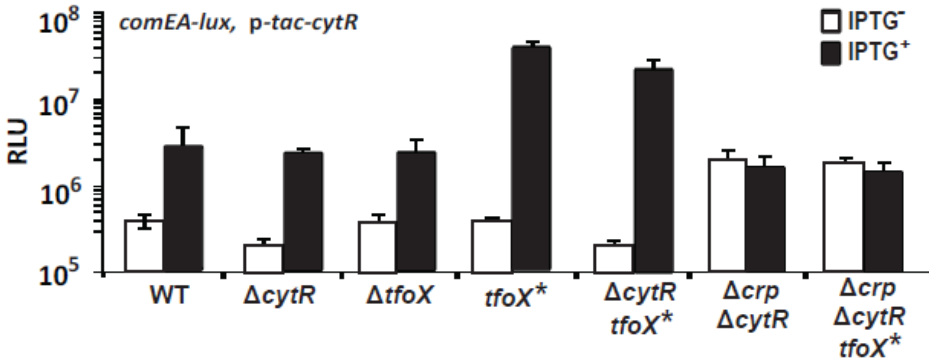

Figure 3.5. CytR overexpression is not sufficient for maximal *comEA* expression. Triplicate cultures of indicated *V. cholerae* strains expressing *cytR* gene under control of an IPTG-inducible *tac* promoter (p-*tac-cytR*) and carrying a plasmid-borne *comEA–lux* reporter were incubated overnight without and with IPTG (white and black bars respectively) and analysed for luciferase production. Bioluminescence is defined as relative light production per OD600 (RLU). Data shown are mean values ± standard deviation for the triplicate cultures from one representative experiment of three performed.

3.4.6. Cytidine is a repressor of natural competence

In *E. coli*, protein-protein interactions between CytR and CRP result in anti-activation of numerous CRP-activated metabolism genes, including *udp* and *cytR* itself (175). CytR is termed the Cytidine Repressor because the accumulation of cytidine is thought to induce conformational changes in CytR that weaken contact with CRP. Prior studies in *E. coli* have shown that cytidine relieves CytR-dependent anti-activation (repression) and permits CRP-dependent activation of nucleoside scavenging genes including *udp* in minimal media lacking glucose (9). Thus, we next tested the role of cytidine scavenging in CytR anti-activation of genes controlling natural transformation in *V. cholerae*. We measured levels of *comEA-lux* expression in *V. cholerae* strains incubated in AB minimal media that was supplemented with chitin to activate *tfoX* expression and to provide the bacteria with a carbon source. Under these conditions where induction of *tfoX* by chitin promotes competence, the presence of 100mM cytidine reduced transcription of *comEA-lux* in WT *V. cholerae* >100-fold, to levels comparable to a *ΔcytR* mutant, which was unresponsive to cytidine addition (Fig. 3.6A).

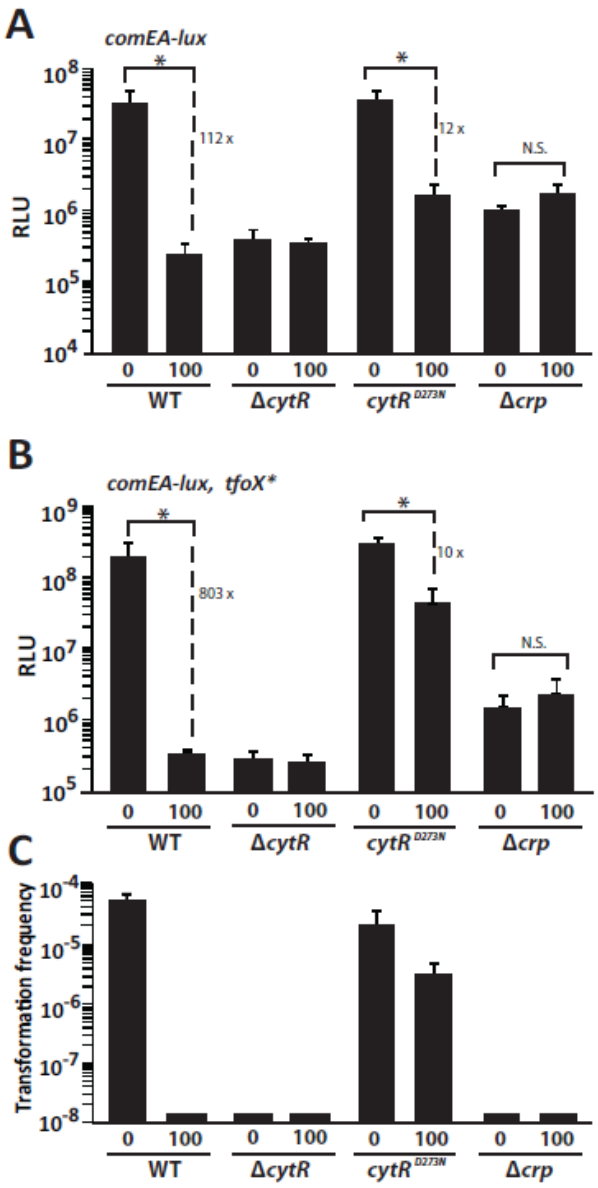

Figure 3.6. Scavenging of cytidine prevents CytR-dependent expression of *comEA–lux.* *V. cholerae* strains carrying *comEA–lux* on a plasmid were incubated overnight in AB minimal media containing a crab shell tile, and supplemented with 100 mM cytidine where indicated. **(A)** Induction of the native *tfoX* allele occurred in response to the chitin tile that also served as a carbon source. **(B)** Constitutive expression of the chromosomal *tfoX* gene controlled by the constitutive *tac* promoter uncoupled *tfoX* induction from the chitin tab provided. Bioluminescence is defined as relative light production per OD600 (RLU). **(C)** Chitin-induced transformation frequency was calculated for each *V. cholerae* strain incubated with extracellular DNA and 100 mM cytidine where indicated in triplicate wells carrying crab shell tabs. The limit of detection is < 10^{-8}. Data shown are mean values _ standard deviation from one representative experiment of three performed. *P < 0.001, N.S.P > 0.05 (t-test).

E. coli CytR with amino acid substitution D281N (corresponding to D273N in *V. cholerae*) (Fig. 3.4C) binds cytidine with three orders of magnitude lower affinity than native CytR and this mutation severely curtails cytidine-mediated disruption of CytR-CRP interactions (9, 10). We constructed a *V. cholerae* strain that carries the corresponding *cytR*^{D273N} allele and compared *comEA* expression in this mutant to the isogenic WT control strain carrying the native *cytR* allele in the presence or absence of 100 mM cytidine. The expression levels of *comEA-lux* in the *cytR*^{D273N} mutant were similar to WT when incubated in AB minimal medium with chitin but lacking exogenous cytidine. However, unlike the wild type strain that experienced an >100-fold reduction in *comEA* in response to cytidine, the *cytR*^{D273N} mutant showed only a slight (12-fold) reduction in *comEA-lux* expression (Fig. 3.6A), indicating that CytR mediates the cytidine response observed in this assay. Expression of the *comEA* reporter in a *Δcrp* mutant, which was already ~10-fold decreased relative to WT strain incubated without cytidine (as in Fig. 3.4D), remained unresponsive to cytidine (Fig. 3.6A), because the absence of CRP is epistatic to both CytR- and cytidine-mediated effects.

To uncouple the role of chitin as a nutrient source and inducer of *tfoX* in our assay, we performed a complementary experiment with strains that expressed the constitutive *tfoX** allele. The expression patterns in the presence of cytidine were similar, even under conditions where *tfoX* was no longer under control of its native promoter (Fig. 3.6B). The effect of cytidine on *comEA* expression in the WT strain carrying tfoX* was enhanced, resulting in a ~800-fold reduction in the presence of cytidine. The remaining *tfoX** strains displayed a similar pattern as in Fig 3.5A, with no effect of cytidine on the Δ*cytR* and Δ*crp* mutant, and a minor (10-fold) reduction in the *cytR*^{D273N} mutant.

Finally, to test whether the presence of exogenous cytidine not only halted *comEA* expression in a CytR-dependent manner, but also prevented DNA uptake, we measured transformation efficiency of corresponding strains carrying the native *tfoX* allele but incubated on chitin. Unlike the WT strain that is highly transformable, the WT strain incubated on chitin with 100 mM cytidine showed an ~10,000-fold defect in transformation, with no DNA uptake recorded, similar to the *ΔcytR* mutant (Fig. 3.6C), which was not transformable without and with 100 mM cytidine. In contrast, transformation frequency of the *cytR*^{D273N} mutant was similar to WT grown without cytidine, and only slightly decreased (10-fold) in the presence of cytidine (Fig. 3.6C). The Δ*crp* mutant that expressed intermediate *comEA* levels that were unresponsive to cytidine addition, was not transformable as expected (Fig 3.6C). Deoxycytidine also severely impaired CytR-dependent transformation in a manner similar to cytidine (Fig. 3.7), suggesting that DNA uptake in *V. cholerae* is responsive to the presence of extracellular nucleosides and deoxynucleosides. Thus, a cytidine-responsive CytR-dependent nucleoside scavenging mechanism, described in *E. coli* (10), appears to be a critical component of a regulatory network controlling natural competence in *V. cholerae*.

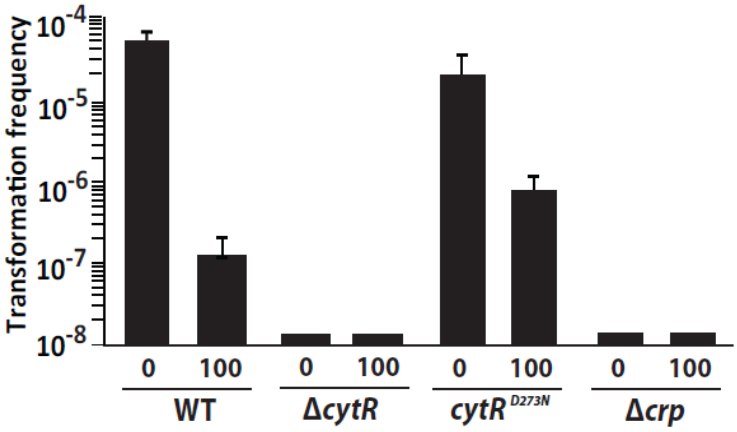

Figure 3.7. **Scavenging of deoxycytidine impairs CytR-dependent natural transformation.** Chitin-induced transformation frequency was calculated for each *V. cholerae* strain with extracellular DNA and 100mM of 2'-deoxycytidine where indicated in triplicate wells carrying crab shell tabs. The limit of detection is 1.0 x 10^{-8}.

3.5. Discussion

Why bacteria become naturally competent to take up DNA is a matter of controversy that has persisted since the pioneering studies of the “transforming principle” by Griffith and later Avery, McLeod and McCarthy who established *Streptococcus pneumoniae* as a model organism for studying natural competence for DNA uptake (7, 67). Numerous studies support the hypotheses that natural competence evolved in bacteria to aid in three major processes: DNA repair, horizontal gene transfer (HGT), and nutrition (for reviews see (57, 146, 163)). However, it is acknowledged that DNA taken up by bacteria may not be used exclusively for one function or another, since extracellular DNA scavenged as nutrient may also be available for recombination onto the chromosome when of sufficient sequence identity (57). Indeed, the nutrition hypothesis has been viewed with particular skepticism as the sole explanation for maintenance of competence systems in bacteria because *Neisseria gonorrhoeae* and *H. influenzae* exclude DNA that is not species-specific, and many bacteria including *B. subtilis* can secrete exoenzymes to degrade extracellular DNA and then utilize nucleoside scavenging transporters for acquisition of the extracellular bases (46, 47, 57, 124). Our results presented here begin to define components of a nucleoside scavenging system in *V. cholerae* that, along with chitin and quorum sensing signaling, alters expression of transformation gene expression. Indeed, features of this emerging network suggest that regulation of *V. cholerae* natural competence has characteristics of pathways described below for both gram negative and gram positive bacteria.

Despite the unique features and regulatory components of the chitin- and QS-induced natural competence system described in vibrios, the general architecture of this regulatory network shares some features with other naturally competent bacteria. In

particular, gram positive *S. pneumoniae* and *B. subtilis* require a peptide-based QS system to regulate a phosphorylation cascade, which induces a regulator (sigma factor) that controls genes for the uptake DNA without sequence preference (47). In contrast, gram negative *N. gonorrhoeae* and *H. influenza*e only take up DNA carrying species-specific uptake sequences; yet do not appear to use QS to mediate this process (36). Interestingly, it has been proposed (57, 171) that *S. pneumoniae* and *B. subtilis* regulate competence in response to species-specific QS AIs to limit competence induction to high cell density conditions that may favor acquisition of "self" and not "foreign" DNA. It is believed that *N. gonorrhoeae* and *H. influenzae*, by contrast, utilize a sequence-based mechanism rather than QS to ensure "sexual isolation". However, such a model is insufficient to explain the *V. cholerae* competence network elucidated here. First, unlike the other gram negative bacteria that regulate competence without QS system input, *Vibrios* appear to be notable exceptions to this generalization. Second, *V. cholerae* lacks uptake sequences and instead appears to take up DNA broadly (165), in contrast to the archetypal gram negative *N. gonorrhoeae* and *H. influenzae*. Finally, the competence network in *Vibrios* shares additional features with gram positive systems by not only utilizing QS signaling but also a regulatory circuit for monitoring nutrient stress. A complex regulatory circuitry in *B. subtilis* coordinates competence and sporulation in response to nutrient cues (68). So too, the CcpA regulator in *S. gordonii*, which orchestrates a CCR similar to that in gram negative bacteria, controls both biofilm formation and natural competence as a response to nutrient depravation (193).

Vibrios commonly form biofilms on biotic chitinous surfaces, such as chironomids and zooplankton molts (72, 169) producing chitinases that allow exploitation of this abundant GlcNAc polymer in an otherwise nutrient-poor aquatic biosphere (108). Initial studies demonstrating that chitin also induced natural competence noted that the presence of

glucose suppressed DNA uptake, which prompted the suggestion that competence in *V. cholerae* was under control of carbon catabolite repression (CCR) (118). Indeed, Blokesch recently confirmed a role for CRP in DNA uptake by *V. cholerae*, although a specific mechanism for the role of CRP in competence was not validated (21).

Cameron and Redfield have proposed a model for γ-proteobacteria (including *E. coli*, *V. cholerae* and *H. influenzae*) that transcription from the promoters of *comEA* and *pilABCD* may be under Sxy (TfoX) control (30). Specifically, *H. influenzae* Sxy (TfoX) is proposed to direct CRP to interact with a competence regulatory element (CRE) sequence (TGCGA-N6-TCGCA) in the *comE1* (*comEA*) and *pilA* promoters, although a precise mechanism has not been revealed to fully explain how CRP and/or Sxy engage at the CRE site (which is remarkably similar to the CRP binding site: TGTGA-N6-TCACA) (147). Inspection of the promoter region of *V. cholerae comEA* that is included in our reporter fusion indicates one potential CRE site (TGCGA-N6-AAGCA); and the *pilA* promoter contains a potential CRP binding site or a CRE (TGAGA-N6-TCAAA), but is not in our reporter fusion (data not shown). Thus it is possible that in *V. cholerae*, as in *H. influenzae,* CRP (via TfoX) directly promotes transcription of competence genes like *comEA*. In addition, our results here also support a role for CRP (via CytR) in indirectly regulating a similar class of genes as described below. The independent contribution of TfoX and CytR could explain why *tfoX* induction (TfoX*) was required for maximal *comEA* expression when *cytR* was overexpressed (Fig. 3.5). Although naturally competent *H. influenzae* does not encode a CytR homolog, our data linking CytR to competence in *V. cholerae* may be useful in discovering why Enterobacteriacaea such as *E. coli, Shigella*, and *Yersinia* species that encode homologs of both TfoX and CytR (147) are not naturally transformable.

In *E. coli*, which is not naturally competent, the CytR regulator has been extensively studied for its role in anti-activation of a set of CRP-dependent nucleoside scavenging genes (including *cytR, udp, deoP, nupG, cdd, tsx, cytX-rot*) that are anti-activated by CytR (for review see (175)). However, BLASTp analysis suggests that CytR-CRP regulated promoters in *V. cholerae* and *E. coli* may be different (data not shown). In *E. coli,* CytR-controlled promoters contain two CRP binding sites, with the exception of *cytR* itself, which is autorepressed and yet contains a single CRP site (137). This is likely the case for *V. cholerae cytR* as well, which appears to contain a single CRP binding site in its promoter region (data not shown). So, while we predict that the *V. cholerae* CytR regulon includes *cytR* and *udp* (Figs. 3.3 and 3.4, (83, 196)), the apparent *V. cholerae deoP* homolog*, deoC*, lacks two CRP sites in its promoter*,* and *V. cholerae* does not encode an obvious *nupG* homolog (data not shown). As a result, we are currently defining the CytR regulon by experimental and computational methods to further define whether CytR has direct or indirect effects on competence gene expression.

Our results presented here are consistent with a model that CytR-CRP interactions have a positive effect on competence gene expression and DNA uptake. However, it is likely that one or more intermediate steps exist between CytR-CRP and the competence genes described here. Only the *udp* promoter contains two CRP binding sites for direct anti-activation, while the promoter regions of *comEA*, *chiA-1* and *pilA* do not have two CRP binding sites (data not shown). While it remains possible that *V. cholerae* CytR may not act identically to its *E. coli* counterpart as an anti-activator, we favor a speculative model supported by our data that CytR and CRP, via protein-protein interactions, interact with the promoter of a putative factor *X*, which in turn represses *comEA*, *chiA-1* and *pilA* (Fig. 3.8). Given such a mechanism, WT *V. cholerae* would result in maximal competence gene expression, a Δ*cytR* mutant would maximally repress competence,

and a Δ*cytR*, Δ*crp* double mutant unable to activate *X* could results in intermediate *comEA* levels (Fig. 3.8). However, since CRP is a pleiotropic regulator, and a *V. cholerae* Δ*crp* mutant displays a growth defect (data not shown, (21, 159)), it is also possible that the intermediate expression observed here for Δ*crp* mutants may be complex and result from changes in a CytR response, as well as consequence due to growth alterations or additional role(s) that CRP may play in directly regulating one or more competence genes. Nonetheless, our epistasis results support that the CytR-mediated effects observed here do not occur in the absence of CRP, consistent with our model that CytR function in *V. cholerae* requires CRP.

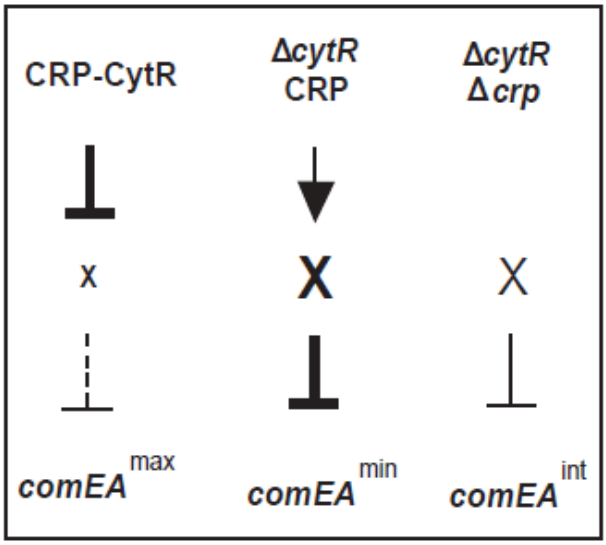

Figure 3.8. A model for the role of a putative repressor X in CytR-dependent anti-activation of the competence gene, *comEA*, in *V. cholerae*. Left: CytR–CRP anti-activation of the X repressor results in maximal expression of *comEA*. **Middle**: In a Δ*cytR* mutant, CRP activation of X results in minimal *comEA* expression. **Right**: In a Δ*cytR,* D*crp* mutant, the lack of CRP activation of X results in intermediate basal *comEA* expression levels.

Haugo & Watnick demonstrated that CytR represses biofilm formation in *V. cholerae* strain MO10, although a direct mechanism linking CytR to biofilm genes was not

revealed (83). Recently, Garavaglia *et al*. have also demonstrated CytR repression of *E. coli* biofilms, as a Δ*cytR* mutant displayed reduced expression of the *csgDEFG* operon, which controls assembly and transport of curli fibers that promote aggregation (65). Modulation of intracellular pyrimidine concentrations appears responsible for the changes in curli expression leading the authors to propose that biofilm gene expression is an indirect consequence of CytR control of nucleoside pools in the cell. It remains possible that CytR functions in a similar indirect manner to control expression of the competence genes in *V. cholerae* and experiments are underway to test this.

In V. cholerae A1552, *cytR* was identified one of ~100 genes positively regulated at least 2.5-fold by *tfoX* induction (118), however, we did not observe an increase in *cytR-lux* expression in *V. cholerae* strain C6706 under similar conditions (data not shown). So too, we showed that maximal *comEA* expression required *tfoX* activation (tfoX*), as cytR overexpression was not sufficient to bypass the need for TfoX (Fig. 3.5). It is possible that TfoX controls transcription of *comEA*, and other competent genes via direct interactions (at CRE sites as indicated above), while CytR plays an additional independent role. Determining the identity of a putative factor X that may be anti-activated by CytR in *V. cholerae* could indeed provide insight into how CytR-CRP mediates its effects on competence. Complementary biochemical, bioinformatics and genomic methods are being developed to identify the set of CytR-regulated targets.

The manner in which HapR influences *V. cholerae* competence genes is likely to be complex. Earlier models predicting a more direct role for HapR exclusively on *comEA* (5, 165) appear insufficient to explain the observations here that *pilA* and *chiA-1* are also regulated like *comEA* in a manner that depends on TfoX, CytR and HapR (Fig. 3.3). Rather than direct interaction of HapR with each promoter (or a unique upstream

regulator for each gene), it is likely that HapR may also impinge on this network by directly controlling a single factor that in turn regulates multiple competence genes. Induction of *cytR* by HapR was not observed in prior studies or in this current study (118), and data not shown). In accordance with this, a strain carrying a constitutive *tfoX** allele still requires *hapR* for *comEA* expression and DNA uptake (Fig. 3.2A and 3.2B); thus like CytR, HapR likely regulates competence in a manner that is TfoX-independent. HapR also regulate *udp* transcription, as a Δ*hapR* mutant relative to an isogenic $HapR^+$ strain is increased for *udp* transcription (Fig. 3.3D), and also slightly increased for *udp* transcript abundance in *V. cholerae* C7258 strain (109). Lo Scrudato & Blokesch recently concluded that HapR does not regulate *pilA* expression, but acknowledge that variations in regulatory circuits of diverse *V. cholerae* strains may result in different interpretations regarding the role of quorum sensing and also CRP (112). QS in *V. cholerae* C6706 controls many genes including several that alter levels of the intracellular second messenger molecule, cyclic dimeric GMP (c-di-GMP), which acts on many targets in the cell (76, 179). HapR-mediated effects on competence may also be an indirect consequence of alterations in this pool of di-nucleotides in response to high cell density conditions. Interplay between c-di-GMP and levels of intracellular and extracellular nucleic acids would suggest complex metabolic changes while *V. cholerae* is in the naturally competent state.

A similar QS system to that described in *V. cholerae* C6706 is used by many members of the *Vibrio* genus (131); and chitin-induced DNA uptake has been demonstrated for several *Vibrios* including *V. parahemolyticus*, *V. fischer*i, and *V. vulnificus* (38, 70, 139). Counterparts to the competence genes and *cytR* are also present in other Vibrios ((14), and data not shown), however a more complete understanding of the network connections between these systems in *Vibrios* is obviously required. Curiously, *V.*

harveyi does not appear to be naturally competent under the assay conditions described here for *V. cholerae* (5), but has been proposed to use CytR to control a regulon important for pathogenesis of marine hosts (144). Naturally competent *V. fischeri* encodes a CytR protein with an amino acid substitution at a position corresponding to F165 in *V. cholerae* (data not shown), suggesting that addition contacts may mediate CytR-CRP interaction in some Vibrios.

As we have proposed previously, the reliance of *V. cholerae* on a genus-wide QS system to control DNA uptake may be a contributing factor sculpting the genome of *V. cholerae*, which has undergone rampant HGT (5, 43). As nucleotide scavenging, via CytR-CRP, appears to halt DNA uptake, as shown here, it may be that the evolutionary role of competence in the Vibrios includes HGT as well as nutrient acquisition. Determining whether extracellular DNA and nucleosides can support growth of *V. cholerae*, as shown for E. coli (60) is an important next step. Uncovering the complex network connections linking TfoX, HapR and CytR to competence will likely contribute to our knowledge of signaling in other naturally competent *Vibrios*, as well as elucidate an expanding role for CytR-based regulation. Defining of the CytR role in *Vibrios* may also be applied to understanding Enterobacteria, like *E. coli,* that encode CytR and many other competence genes and appear to have genetic differences that limit DNA uptake (147, 158).

3.6. Acknowledgements

We thank B. Duke and M. Nellesson for assistance with luciferase assays. V. Rajan and M. Borodovsky provided support for genome analysis. This study was funded by National Science Foundation grants (MCB-0919821 and MCB-MCB-1149925) to B.K.H.

CHAPTER 4

CLINICAL ISOLATES OF *V. CHOLERAE* FROM HAITI PATIENTS ARE IMPAIRED FOR NATURAL COMPETENCE

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4.1. Abstract

In October 2010, nearly 10 months after a devastating earthquake, Haiti was stricken by epidemic cholera. In 2011, whole genome sequencing efforts attributed the origin of the outbreak to accidental single-source introduction of an O1 strain of *Vibrio cholerae* by United Nations peace keepers from Nepal, where cholera outbreaks are not uncommon. In 2012, it was then reported that two distinct populations of *V. cholerae* coexisted in Haiti early in the epidemic, both the O1 and non-O1 *V. cholerae*, which is more often found in environmental settings. It was hypothesized that both *V. cholerae* O1 and environmental strains might contribute to spread of cholerae disease with the non-O1 strains also serving as a cholera toxin reservoir for potential horizontal gene transfer (HGT) and rapid evolution of O1 strains. However, an on-going study, which includes extensive whole genome sequencing of a set of Haiti clinical strains collected from different time points and locations, showed no demonstrable HGT. Indeed, we demonstrate that, consistent with genomic analysis of this set of strains, each Haiti isolate tested was impaired for transformation by laboratory HGT assays, despite a functional quorum-sensing system, which is often defective in other non-transformable *V. cholerae* isolates. Further studies will define the genetic mutation(s) that render the Haiti strains defective for natural transformation and help to elucidate the complex network of *V. cholerae* natural competence.

4.2. Introduction

Cholera is a disease endemic in many developing countries, and its causative agent, *Vibrio cholerae*, is a common inhabitant of estuaries and river systems worldwide (49). The recent outbreak of cholera in Haiti is a tragic reminder of how rapidly a cholera epidemic can emerge and spread through a population. Cholera was first detected in Haiti in October of 2010 and has resulted in 604,634 cases and 7,436 deaths over the first two years (15).

The absence of a previously recorded history of epidemic cholera in Haiti (96) raised interest in understanding the source of the outbreak. Identical pulsed field gel electrophoresis patterns for 13 bacterial isolates recovered from Haitian patients with cholera led to a conclusion that the Haitian isolates derived from a strain similar to *V. cholerae* isolated in South Asia (31). Later studies have attributed the Haiti epidemic to the introduction of a single *V. cholerae* strain (of the serogroup O1) from Nepal on the basis of both epidemiological and genetic data (42, 62, 87). However, the identification of non-O1 *V. cholerae* strains in Haitian patients suggested potential contribution of these strains to disease in Haiti (82).

Being primarily an aquatic bacterium, *V. cholerae* can persist indefinitely in rivers, estuaries, and coastal regions without any need for human passage. Within environmental reservoirs *V. cholerae* commonly forms biofilms on abiotic and biotic surfaces, such as chitinous chironomids and the chitinous exoskeleton of copepods (72, 169). In *V. cholerae,* growth on chitinous surfaces, in conjunction with functional quorum sensing (QS) and a nucleoside scavenging system, initiates a natural competence program that allows bacteria to take up free DNA from the environment and incorporate

it into the chromosome (4, 5, 112, 118). Competence for genetic transformation represents one mode of horizontal gene transfer (HGT), along with conjugation and transduction, which enables bacteria to acquire genetic material from other sources. It is believed that *V. cholerae* is an extremely versatile bacterium that can quickly adapt to new environments by acquiring new genetic material from the environment (43). In the present study, we examine the ability of a set of twelve Haiti clinical strains collected from different time point and locations to take up extracellular antibiotic-marked DNA and to become transformed. On the basis of this analysis, we discuss the potential role of HGT and the evolutionary dynamics of this deadly pathogen in Haitian cholera epidemic.

4.3. Experimental procedures

4.3.1. Bacterial isolates and culture conditions

Bacterial isolates analyzed in this study were received from Cheryl L. Tarr at the Centers for Disease Control and Prevention (CDC) and are listed in Table 4.1. Clinical O1 isolate C6706, the isogenic Δ*hapR* mutant, and the C6706 derivative carrying *kanR* at the *lacZ* site were from the Hammer lab strain collection. Transposon mutagenesis of C6706 and the Haiti isolates was performed as described (4). Strains were incubated at 37°C on Luria-Bertani (LB) agar, and in LB broth with shaking. Artificial sea water (ASW; Instant Ocean) was used for chitin-induced natural transformation assays as described previously (5, 118). Antibiotics (Fisher BioReagents) chloramphenicol (Cm) and kanamycin (Kan) were used at concentrations of 10 and 100 μg ml^{-1}, respectively. Expression of the *tfoX* gene encoded on p*tfoX* was induced with 0.5mM isopropyl-b-D-thiogalactopyranoside (IPTG; Fisher BioReagents).

Table 4.1. Clinical isolates from Haiti patients received from Centers for Disease Control and Prevention, Atlanta.

Strain ID	Geographical origin	Date of origin	Description*
2010EL-1786	St. Marc Hospital, Artibonite, Haiti	10/19/2010	O1 OGET
2010EL-1799	St. Nicholas Hospital, Artibonite, Haiti	10/21/2010	O1 OGET
2010EL-2026	Bon Samaritain Hospital, Cazale, West, Haiti	11/09/2010	O1 OGET
2011EL-1818	Cholera Treatment Center, Tabarre, West, Haiti	06/06/2011	O1 OGET
2011EL-1841	Medecins Sans Frontieres, Carrefour, West, Haiti	06/09/2011	O1 OGET
2011EL-2319	Anse-à-Galets, La Gonave, West, Haiti	10/11/2011	O1 OGET
2011EL-2320	Gonaives, Artibonite, Haiti	10/11/2011	O1 OGET
2011EL-2321	Cholera Treatment Center, Oriani, West, Haiti	10/12/2011	O1 OGET
2011EL-2322	Centre Hospitalier de Pernier, Pernier, West, Haiti	10/12/2011	O1 OGET
2011EL-2323	Departement Sanitaire du Sud Est, Sud Est, Haiti	10/12/2011	O1 OGET
2012EL-1410	Artibonite, Haiti	03/12/2012	O1 INET
2011EL-1300	Cholera Treatment Center, Les Cayes, South, Haiti	02/12/2011	O1 OGET

*Descriptions: OGET, Serotype Ogawa Biotype El Tor; INET, Serotype Inaba Biotype El Tor.

4.3.2. Chitin-induced natural transformation assay for HGT

As described (5, 118), sterile crab shells in triplicate wells were inoculated with each *V. cholerae* strain in a 12-well plate and provided with 2 ug of genomic DNA (gDNA) marked at the *lacZ* locus with a kanamycin resistance (*kanR*) gene. Following a 24 h incubation, attached cells were harvested and plated to quantify transformation frequency (T.F.) defined as *kanR* CFU ml^{-1}/total CFU ml^{-1} and the average value of T.F. for each sample was calculated. Independent experiments were performed in triplicates.

In Table 4.2 each strain was provided with donor gDNA from a C6706 derivative with *kanR* at the *lacZ* locus. In Table 4.3, twelve pools of donor gDNA were generated from >1000 tn*5*(*kanR*) mutants of each isolate; and each pool was used to transform that same isolate and C6706.

4.3.4. Bioluminescence assay

As described (121) each strain carrying a lux-based reporter plasmid (pBB1 or p*comEA*) was grown in triplicate cultures overnight at 37^{o}C overnight with appropriate antibiotics and IPTG where noted. Bioluminescence and absorbance were measured thereafter. Relative Light Units (RLU) are defined as counts $min^{-1}ml^{-1}/OD_{600}$. Shown are mean values +/- standard deviation from one representative experiment of three performed.

4.4. Results and Discussion

HGT is one of the major driving forces of bacterial evolution (134); however, little is known about the rate of HGT in natural populations over short time scales. A previous study of *V. cholerae* from Haiti reported accumulation of diversity over the initial three weeks of the epidemic (82). Because these results are inconsistent with later analyses of an overlapping but expanded set of genomes (Katz *et al*, submitted), other hypotheses were considered that could explain the observed absence of HGT.

One method of HGT in *Vibrios*, natural transformation, occurs on chitinous surfaces and requires quorum sensing (QS) (i.e. the HapR QS transcription factor) (5, 118). Thus, we sought to test whether the Haiti *V. cholerae* isolates were naturally competent and capable of taking up and incorporating foreign DNA. Specifically, we tested twelve Haiti isolates, along with competent-proficient strain C6706 and a competent-deficient

isogenic Δ*hapR* derivative (5), to determine whether the Haiti strains were naturally competent (Table 4.2). To do that each strain was provided with donor gDNA from a C6706 derivative with *kanR* at the *lacZ* locus (or C6706::*kanR* gDNA), and transformation frequency (T.F.) was defined as *kanR* CFU ml^{-1}/total CFU $ml^{-}1$. The mean transformation frequency of triplicate wells of C6706 was 1.3 x 10^{-5} and consistent with previous observations (5). As expected, no *kanR* CFUs were detected (<DL) for C6706 Δ*hapR*, so the estimated T.F. was 6.5 x 10^{-9}, indicating a competence defect of at least 1000-fold (Table 4.2). Note that T.F. varies for each strain because the total CFU varies for each of the three wells (Table 4.2). Similarly, no *kanR* CFUs were detected (< DL) with any of the twelve Haiti isolates provided with C6706::*kanR* gDNA; thus each Haiti isolate, like C6706 Δ*hapR,* appeared to be severely impaired for uptake of C6706 DNA.

Table 4.2. Haiti isolates are defective for transformation with C6706 gDNA marked by *kanR* at *lacZ* locus.

Recipient strain	T.F.	Range	Fold Reduction
C6706	< 1.3 x 10^{-5}	7.8 x 10^{-6} - 1.9 x 10^{-5}	1
C6706 Δ*hapR*	< 6.5 x 10^{-9}	<DL	> 2001
2010EL-1786	< 3.8 x 10^{-9}	<DL	> 3428
2010EL-1799	< 2.5 x 10^{-9}	<DL	> 5200
2010EL-2026	< 1.9 x 10^{-9}	<DL	> 6948
2011EL-1818	< 1.6 x 10^{-9}	<DL	> 8196
2011EL-1841	< 1.5 x 10^{-9}	<DL	> 8392
2011EL-2319	< 5.4 x 10^{-9}	<DL	> 2399
2011EL-2320	< 1.3 x 10^{-9}	<DL	> 9588
2011EL-2321	< 1.4 x 10^{-9}	<DL	> 9508
2011EL-2322	< 3.6 x 10^{-9}	< DL - 8.6 x 10^{-9}	> 3621
2011EL-2323	< 1.8 x 10^{-9}	<DL	> 7223
2012EL-1410	< 7.3 x 10^{-10}	<DL	> 17648
2011EL-1300	< 9.5 x 10^{-10}	<DL	> 13635

T.F. Average transformation frequency from triplicate samples.
DL. Detection limit was <1.0 x 10^{-8} for all experiments.

We hypothesized that non-transformable phenotype of Haiti isolates might be due to active restriction modification system that prevents bacteria from incorporation of foreign DNA derived from C6706 (104). To test that, twelve pools of donor gDNA were generated by transposon mutagenesis. For example, one Haiti isolate was mutagenized with a tn*5*(*kanR*) transposon, >1000 kanR colonies were pooled from agar plates, and then gDNA was extracted. In total, a pool on *kanR*-marked gDNA was obtained for each isolate in this manner, as well as for C6706. C6706 was readily transformed by its own gDNA or gDNA derived from each Haiti strain, with T.F. comparable to that observed prior (Table 4.2, 4.3). As expected, no *kanR* CFUs were detected for C6706 Δ*hapR* incubated with DNA from any isolate tested (Table 4.3, data not shown), which is consistent with prior results (Table 4.2). As observed, each Haiti isolates was severely impaired for the ability to take up DNA derived from itself (Table 4.3). These results suggest that restriction modification system does not prevent the transformation of Haiti isolates, and the defect is likely caused by one or more mutations in the competence apparatus or in the signaling systems essential for natural competence.

Table 4.3. Haiti isolates are defective for transformation with self-derived tn*5*(*kanR*) gDNA.

gDNA	Recipient strain	T.F.[a]	Range	Recipient strain	T.F.[b]	Range	Fold Reduction
2010EL-1786	2010EL-1786	$< 5.1 \times 10^{-9}$	<DL	C6706	5.3×10^{-6}	4.7×10^{-6}-5.7×10^{-6}	> 1046
2010EL-1799	2010EL-1799	$< 2.1 \times 10^{-9}$	<DL	C6706	1.8×10^{-5}	1.1×10^{-5}-1.9×10^{-5}	> 8462
2010EL-2026	2010EL-2026	$<1.7 \times 10^{-9}$	<DL	C6706	6.0×10^{-6}	1.8×10^{-6}-1.0×10^{-6}	> 3484
2011EL-1818	2011EL-1818	$<2.1 \times 10^{-9}$	<DL-2.5×10^{-9}	C6706	1.4×10^{-5}	8.8×10^{-6}-1.9×10^{-5}	> 6842
2011EL-1841	2011EL-1841	$<8.1 \times 10^{-9}$	<DL-1.3×10^{-8}	C6706	1.9×10^{-5}	1.2×10^{-5}-2.5×10^{-5}	> 2314
2011EL-2319	2011EL-2319	$<2.0 \times 10^{-9}$	<DL	C6706	1.4×10^{-5}	5.6×10^{-6}-1.7×10^{-5}	> 7087
2011EL-2320	2011EL-2320	$<1.9 \times 10^{-9}$	<DL	C6706	1.7×10^{-5}	1.1×10^{-5}-1.8×10^{-5}	> 8826
2011EL-2321	2011EL-2321	$<5.4 \times 10^{-9}$	<DL-9.9×10^{-9}	C6706	1.9×10^{-5}	9.3×10^{-6}-6.2×10^{-5}	> 3437
2011EL-2322	2011EL-2322	$<5.7 \times 10^{-9}$	<DL-3.3×10^{-9}	C6706	1.0×10^{-5}	1.1×10^{-6}-2.3×10^{-5}	> 1789
2011EL-2323	2011EL-2323	$<3.7 \times 10^{-9}$	<DL-1.9×10^{-9}	C6706	1.1×10^{-5}	4.7×10^{-6}-1.1×10^{-5}	> 2966
2012EL-1410	2012EL-1410	$<4.2 \times 10^{-9}$	<DL	C6706	4.8×10^{-5}	3.7×10^{-6}-5.6×10^{-6}	> 1161
2011EL-1300	2011EL-1300	$<1.6 \times 10^{-9}$	<DL	C6706	9.4×10^{-6}	5.7×10^{-6}-1.4×10^{-5}	> 5960
2011EL-1300	C6706 Δ*hapR**	$<6.4 \times 10^{-9}$	<DL	C6706	9.4×10^{-6}	5.7×10^{-6}-1.4×10^{-5}	> 1476

T.F. Average transformation frequency from triplicate samples.
DL. Detection limit was $<1.0 \times 10^{-8}$ for all experiments.
Fold reduction = TF^a/TF^b.
*C6706 Δ*hapR* recipient was transformed with gDNA from 2011EL-1300.

Several sequenced and well-studied *V. cholerae* strains are known to have a frameshift mutation in a *hapR* gene resulting in a loss of QS capability (97, 118). Since it is known that HapR$^-$ mutants are impaired for natural competence (5, 118), we reasoned the Haiti strains may have defects in the QS system (i.e. mutant for HapR), therefore, lacking one of the essential inputs that induces natural competence in *V. cholerae* (5, 118). To test that, each isolate was conjugated with *E. coli* to introduce a well described reporter plasmid (pBB1) that carries the *V. harveyi* luciferase (*lux*) operon (121). The l*ux* operon is expressed only in *V. harveryi* and *V. cholerae* strains that have a functional QS system and thus can produce the transcription factor HapR at high cell density. Measurement of pBB1 expression in Haiti isolates confirmed that each isolate, like C6706, expressed *hapR* and was QS-proficient (Fig. 4.1), unlike a ∆*hapR* mutant. Specifically, each of the twelve isolates expressed at least as much luciferase as the positive control strain C6706. The negative control strain, an isogenic Δ*hapR* derivative of C6706, expressed ~1000-fold less light than positive control, which corresponds to the limit of detection for this assay. Thus, the Haiti strains appear to be limited in the ability to acquire new genetic material through transformation, but this is not due to QS-deficiencies identified in other non-transformable *V. cholerae* isolates (118) (Hammer & Bernardy, unpublished results).

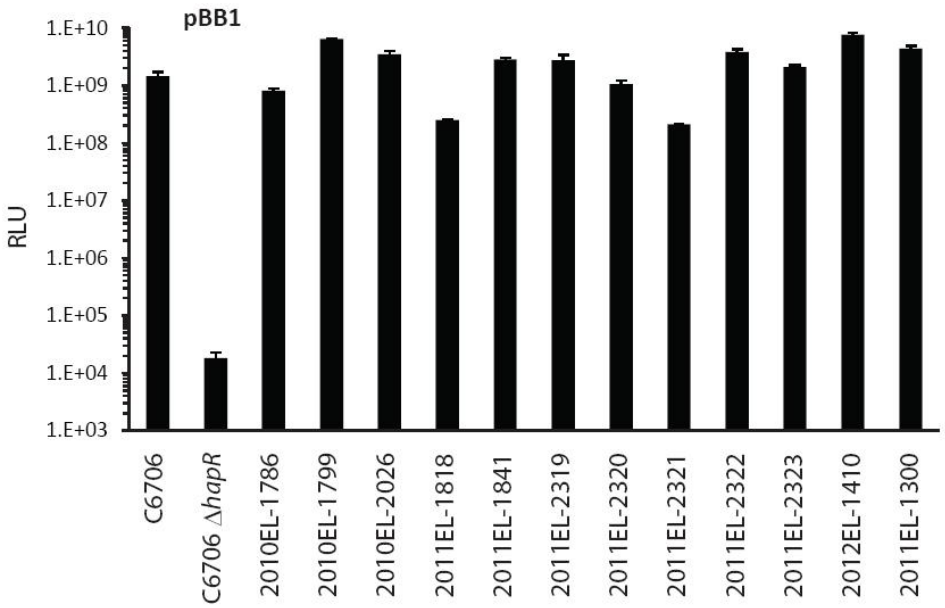

Figure 4.1. Each Haiti isolate is quorum sensing proficient. Triplicate overnight high cell density cultures of each strain were measured for the luciferase levels and optical density (OD600); Relative Light Units (RLUs) were calculated as counts per min^{-1} ml^{-1}/OD_{600}. Data shown are mean values ± standard deviation from one representative experiment of three performed.

Along with HapR, TfoX regulator expressed in response to chitin is also required for positive control of a competence gene, *comEA,* as well as several other genes of the competence apparatus for DNA uptake (118). To test weather Haiti isolates express *comEA*, we introduced into each isolate a transcriptional reporter gene fusion of *comEA* to the luciferase operon (*comEA-lux*). In C6706, the expression of *comEA-lux* in the absence of chitin requires an IPTG-inducible *tfoX* plasmid (p*tfoX*) (5). As described previously, the *V. cholerae* C6706 reference strain highly expressed *comEA*, while an isogenic Δ*hapR* derivative of C6706 was ~100-fold reduced in *comEA* expression (Fig. 4.2, black bars) (5). When TfoX was induced (+IPTG), six out of twelve Haiti isolates (2010EL-2026, 2011EL-2319, 2011EL-2320, 2011EL-2323, 2012EL-1410, 2011EL-

1300) expressed *comEA* at levels similar to *V. cholerae* C6706. In four Haiti isolates (2010EL-1786, 2011EL-1818, 2011EL-1841, 2011EL-2322) *comEA* expression was ~10-fold reduced compare to C6706, and two strains (2010EL-1799, 2011EL-2321) showed no detectable *comEA* production (Fig. 4.2, black bars). As expected, in the absence of TfoX induction, *comEA* expression was reduced to the limit of detection in all strains (including C6706) confirming the requirement of TfoX for *comEA* transcription (Fig. 4.2, grey bars). Although the factors connecting TfoX to all of the competence genes are not yet known, the results demonstrate that these connections are likely intact in at least 10 out of 12 Haiti strains. Together with previous observations (Tables 4.1, 4.2 and Fig. 4.1) two isolates (2010EL-1799 and 2011EL-2321) appear to be defective in *comEA* expression despite TfoX induction. It is possible, for example, that 10 Haiti isolates that expressed *comEA* in response to TfoX may have a mutation in unknown factor(s) upstream of TfoX, which result in TfoX induction in response to chitin (Fig. 1.2). We are currently determining whether IPTG-inducible *tfoX* expression not only induces *comEA*, but is sufficient to restore DNA uptake by each Haiti isolate. Future collaborations with the CDC include plans to identify the mutation(s) present in each Haiti strain but absent from C6706 responsible for impaired transformation.

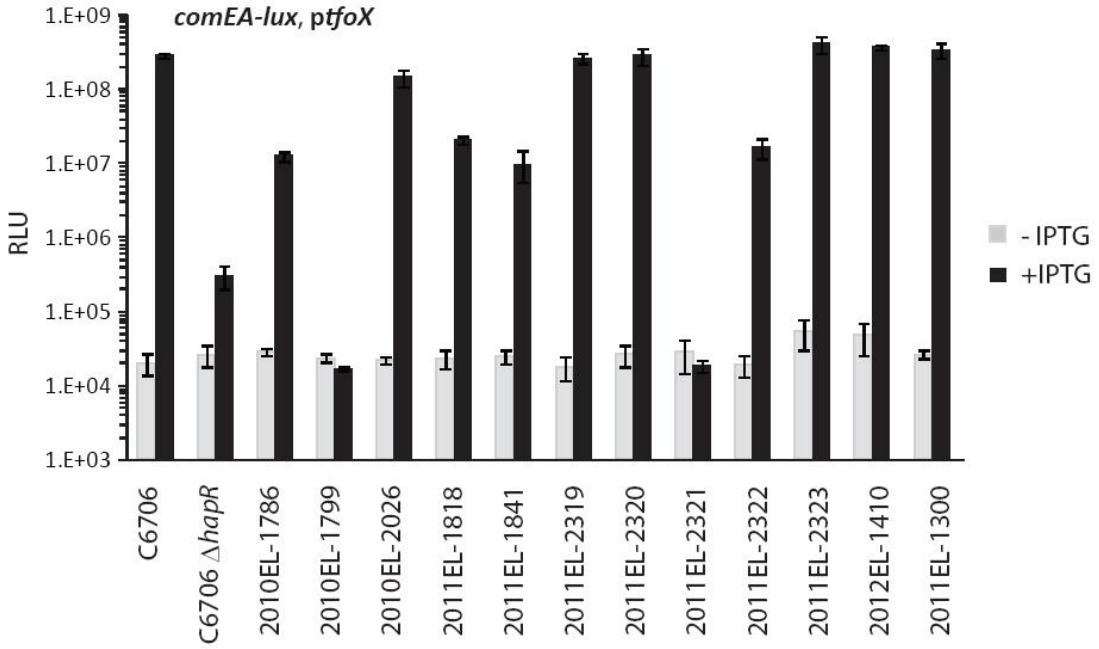

Figure 4.2. Expression pattern of a competence gene, *comEA,* in Haiti isolates. *V. cholerae* strains carrying a *comEA-lux* plasmid and an IPTG-inducible *tfoX* plasmid were incubated with (black bars) or without IPTG (grey bars). Triplicate overnight high cell density cultures of each strain were measured for the luciferase levels and optical density (OD600); Relative Light Units (RLUs) were calculated as counts per min^{-1} ml^{-1}/OD_{600}. Data shown are mean values ± standard deviation from one representative experiment of three performed.

The data presented in this report shows that the Haiti outbreak strains are significantly less competent under laboratory conditions than clinical reference isolate C6706. This observation supports the hypothesis that O1 strains causing the cholera outbreak in Haiti did not exchange DNA with other non-O1 strains in Haiti (42), and are severely impaired for the ability to exchange DNA via natural competence. Nevertheless, it remains possible that *V. cholerae* isolates defective for transformation in lab settings may be still naturally competent in nature. However, no such strains have yet been described. We also note that the low rates of transformation would not affect HGT via other mechanisms such as phage transduction or conjugation.

Natural transformation of *V. cholerae* is coordinated by multiple signaling systems (4, 112). However, sequence analysis of the known components of these regulatory pathways in the 12 poorly-transformable isolates described here (Lee *et al,* 2013, submitted) support an interpretation that these systems are likely intact. We showed that each isolate was QS-proficient and capable of metabolizing chitin as its sole carbon source in the HGT assays. Further genome comparison and genetic studies will help us to find the genetic mutation(s) that render the Haiti strain defective at HGT via natural transformation. These studies suggest that one or more mutations that have impaired the ability of the introduced *V. cholerae* strain to take up DNA by natural competence, may have compromised HGT between this strain and non-O1 strains in Haiti. Identifying this mutation will contribute to the understanding of the role of transformation in genome evolution and fitness of this human pathogen.

4.5. Acknowledgements

We thank Cheryl L. Tarr and Lee Katz at the the Centers for Disease Control and Prevention (CDC) for providing us with Haiti clinical isolates studied here and for the opportunity to collaborate on this project.

CHAPTER 5

CONCLUSIONS AND RECOMMENDATIONS

The ability to sense and respond to environmental signals is critical for survival and adaptation by bacteria. In *Vibrio cholerae*, natural competence for DNA uptake is coordinated by three extracellular signals: chitin, quorum sensing (QS) autoinducer molecules, and extracellular cytidine. Each signal triggers the production of a specific regulator (TfoX, HapR, CytR, respectively), which in concordance with other regulators induces the expression of genes required for competence. Along with conjugation (via plasmids) and transduction (via bacteriophages), competence for genetic transformation is one of horizontal gene transfer (HGT) mechanisms among marine *Vibrios* that may be responsible for rapid genetic change in *V. cholerae*, as reported recently for a collection of Haiti strains.

The research detailed in this dissertation aimed to define regulatory components connecting extracellular signals to natural competence in *V. cholerae*. First, I demonstrated that QS molecules produced by *Vibrios* within multi-species chitinous biofilms are required for DNA uptake by *V. cholerae* reference strain C6706, confirming the critical role of QS signals in HGT. Second, using the transposon-mutagenesis of C6706, I identified a novel positive regulator of competence, CytR, only studied prior in *Escherichia coli* as a regulator of nucleoside scavenging. Third, I tested a series of *V. cholerae* clinical isolates from the recent outbreak in Haiti, and determined that each is severely impaired for transformation by laboratory methods used. I also confirmed that each Haiti isolate was QS-proficient, unlike several *V. cholerae* strains described prior that are defective for DNA uptake due to QS-deficiency. CDC collaborators used comparative genomics methods described elsewhere to show that all known genes for

the competence apparatus and regulatory pathways appear conserved in the Haiti isolates. These results are consistent with the work described here uncovering a regulatory network in which additional factors remained to be identified.

The results of this study suggest several areas of future research:

- Many components promoting DNA uptake in *V. cholerae* have been discovered, however more factors remain to be identified. Namely, TfoX, HapR, and CytR are not predicted to directly bind competence gene promoters, thus additional factors likely exist connecting each regulatory protein to DNA uptake genes. To define direct and indirect targets of each regulator, multiple approaches will be used including RNA-seq and ChIP-seq, as well as comparative genomics.

- In this study a novel CytR-dependent nucleoside scavenging system was defined that participates in DNA uptake via the competence apparatus. Genetic evidence suggests that the regulatory pathways of CytR control in *V. cholerae* may not be identical to that in *E. coli* (Antonova, Bernardy, Watve, and Hammer, unpublished). The mechanism of transport for cytidine and other nucleosides across the outer and inner membranes is still unknown in *V. cholerae*, and how this contributes to dsDNA uptake is a focus of future research. Further studies will elucidate the connections between nucleoside uptake and dsDNA uptake via the competence system.

- *V. cholerae* isolates from Haiti epidemics possess an unusual phenotype being defective for DNA uptake in spite of intact QS system, which has not been described prior. With assistance from CDC colleagues, whole genome sequencing comparing the Haiti isolates to competent reference strain C6706, followed by

genetic studies, will define the genetic mutation(s) that render the Haiti strains defective for natural transformation. Such collaborative studies will elucidate new features of the complex network of *V. cholerae* DNA uptake mechanism.

The findings discussed in this manuscript advanced the scientific understanding of processes contributing to DNA uptake in *V. cholerae*. Because natural competence for DNA uptake is one important mechanism of HGT, these studies have begun to define the role of natural competence in genome evolution and fitness of the *Vibrio* bacteria. How *Vibrio cholerae* emerges to cause outbreak continues to be an area of intense study, perhaps the work described here and future studies that arise from these findings will lead to a better understanding of the contribution of DNA uptake for this important human pathogen.

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